Pesticide Risk Assessment for Pollinators

Proceedings from a SETAC Pellston Workshop

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Chapter 1 Introduction

Worldwide declines in native and managed pollinators have led to an increased global dialogue and focus concerning the potential factors that may be causing these declines. Although a number of factors have been hypothesized as potential contributors to pollinator declines, at this time, no single factor has been identified as the cause. The available science suggests that pollinator declines are a result of multiple factors which may be acting in various combinations. Research is being directed at identifying the individual and combined stressors that are most strongly associated with pollinator declines. Pesticide use is one of the factors under consideration.

In an effort to further the global dialogue, the Society of Environmental Toxicology and Chemistry (SETAC) held a Pellston Workshop[1] to explore the state of the science on pesticide risk assessment for pollinators. The proposal for this SETAC Workshop was developed by a steering committee (hereafter referred to as the Steering Committee) comprised of members from government and nongovernmental organizations who were interested in advancing the science to understand the effect of pesticides on non-target insects. Workshop participants were tasked to advance the current state of the science of pesticide risk assessment by more thoroughly vetting quantitative and qualitative measures of exposure and effects on the individual bee, and on the colony. In doing so, the Workshop aimed to synthesize the global understanding and work that has, thus far, taken place, and to move toward a harmonized process for evaluating and quantitatively characterizing risk to pollinators from exposure to pesticides; and, to identify the data needed to inform that process. The Workshop focused on four major topics:

1. design/identify testing protocols to estimate potential exposure to bees from pesticide residues in pollen, nectar, as well as exposure through other routes exposure;
2. design/identify testing protocols to measure effects of pesticides to developing brood and adult honey bees at both the individual and colony level;

[1] The first Pellston Workshop was held in 1977 to address the needs and means for assessing the hazards of chemicals to aquatic life. Since then, many workshops have been held to evaluate current and prospective environmental issues. Each has focused on a relevant environmental topic, and the proceedings of each have been published as a peer-reviewed or informal report. These documents have been widely distributed and are valued by environmental scientists, engineers, regulators, and managers because of their technical basis and their comprehensive, state-of-the-science reviews. The first four Pellston workshops were initiated before the Society of Environmental Toxicology and Chemistry (SETAC) was effectively functioning. Beginning with the 1982 workshop, however, SETAC has been the primary organizer and SETAC members (on a volunteer basis) have been instrumental in planning, conducting, and disseminating workshop results. Taken from: http://www.setac.org/node/104

3. propose a tiered approach for characterizing the potential risk of pesticides to pollinators; and
4. explore the applicability of testing protocols, used for honey bees (*Apis* bees), to measure effects of pesticides and pesticide risk to native (non-*Apis)* bee species.

Although the term "pollinators" encompasses a broad number of taxa, for the purposes of this SETAC Workshop and its proceedings, the term "pollinators" refers specifically to subspecies and strains of *Apis mellifera* that originated in European (i.e., the honey bee) and other (non-*Apis mellifera*) bees. The Workshop built upon the numerous efforts of different organizations, regulatory authorities, and individuals, both nationally and internationally, aiming to better understand the role and effect(s) of pesticide products on native and honey bees[2].

<u>Workshop Balance and Composition</u>

Similar to other timely and relevant scientific issues addressed by SETAC Pellston Workshops, the issue of pollinator protection is of high interest to scientists employed by governments, business, academia and non-governmental organizations. For this reason, SETAC requires that its workshops be similarly balanced. The Workshop on Pesticide Risk Assessment for Pollinators represented an exceptionally diverse composition by both (employer) sector, and by geography. The forty eight participants (35 panelists and 13 Steering Committee members) included individuals from industry, non-governmental organizations, federal and state governments, beekeepers, and academia and represented five continents (South America, Europe, Australia, North America, and Africa).

This Proceedings of the Workshop on Pesticide Risk Assessment for Pollinators has several sections:

- Chapters 2 through 6 provide background and overview of key elements such as bee biology, ecological risk assessment overview, and protection goals.

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[2] USDA Technical Working Group Report on Honey Bee Toxicity Testing, July 8 and 9, 2009;[HYPERLINK "http://www.aphis.usda.gov/plant_health/plant_pest_info/honey_bees/downloads/twg_report_july_2010.pdf"]
International Commission for Plant-Bee Relationships 10th International Symposium, 2009;[HYPERLINK "http://www.uoguelph.ca/icpbr/pubs/2008%20ICPBR%20symposium%20archives%20Pesticides.pdf"]

- Chapters 7 through 10 capture recommendations by the Workshop on the elements of exposure assessment, effects assessment (laboratory effects and field effects), and risk assessment.
- Chapters 11 through 14 capture discussion around statistical analysis, modelling, risk management, and research needs.

Pollinators, and the honey bee in particular, have been identified as a valued group of organisms because of the services they provide to agriculture and to ecosystem biodiversity. While both native and managed bees contribute to crop pollination, most of the current knowledge of the side-effects on pollinators is in relation to the honey bee. Since it is not possible to test all species, regulatory authorities rely on surrogate species, such as the honey bee, to represent major taxa. Therefore, it is important to understand the ecology and biology of the test organism.

Chapter 2 Overview of the Honey Bee

Jeff Pettis

A key goal of regulatory authorities is to protect non-target organisms from potential adverse effects of pesticides. As it is not possible to test all species, the pesticide risk assessment framework relies on surrogate species to represent major taxa, including insect pollinators. The European honey bee (*A. mellifera*), among the many different bee species, is a desirable surrogate test species in that it is both a commercially valued organism and is also adaptable to laboratory research. In many countries, such as Canada, and United States, the honey bee is used as a surrogate for many other non-target terrestrial insects and for insect pollinators. While honey bees are frequently subject to collateral effects from the use of pesticides in crop production, they are also the beneficiaries of pesticide applications, as beekeepers routinely employ registered pesticides to manage pest problems that occur in managed hives. The in-hive use of pesticides by beekeepers and the potential exposure of bees to environmental mixtures of pesticides used in agriculture coupled with the complex social organization/biology of bees can complicate pesticide risk assessment. Therefore, it is important to understand the ecology and biology of both the surrogate test organism and those species it is intended to protect.

Overview of Honey Bee Biology

From a risk assessment perspective, there are several aspects of honey bee biology which are important to consider as they potentially impact the toxicity studies required, as well as the approach for evaluating potential risks. Colony growth and survival are dependent on the collective actions of individuals that perform various critical tasks; therefore, honey bee colonies act collectively as a "superorganism". The different castes of bees within the hive structure have different functions which can result in differential exposure in terms of duration, magnitude and mode (direct versus indirect, secondary exposure). The survival of an individual bee may be of little consequence as colonies typically have a 10-30% reserve of workers, which reflects and accommodates the high turn-over rate (of the individual) and flexibility of the colony to adapt to its environment. An examination of the roles of various castes within the hive and the implication for risk assessments follows.

A honey bee colony is made up of one queen, several drones, thousands of workers and many immature bees in various stages of development (eggs, larvae, pupae). Worker bees are sexually undeveloped females and constitute the vast majority of the adults in a colony. All the work inside and outside the colony is done by worker bees. Older workers forage outside the hive for pollen and nectar, and thus are vulnerable to contact exposure to pesticides during foraging, as well as dietary exposure during collection/ingestion of pollen and nectar. Workers also serve as a vector for bringing contaminants back to the hive. Young workers clean cells and attend brood whereas middle-aged workers do a variety of tasks mainly within the hive. Both young and middle-aged workers can have secondary exposure to pesticides through contaminated food brought back to the hive. Each colony has a single queen. Once she mates with drones, the queen returns to the hive to begin the task of egg-laying; she will lay up to 1200 eggs per day for several years. The queen performs no other work in the hive, and continues to be fed royal jelly throughout her lifespan. Drones are male bees whose sole function in the hive is to serve as sperm donors for new queens. Like younger and middle-aged workers, queens and drones can have secondary exposure to pesticides through contaminated food brought back to the hive or intentionally used in the colony by beekeepers.

Inputs by worker bees into the colony include pollen, nectar, water, and plant exudates (*e.g.*, sap) used to make propolis. Pollen is used as the source of protein. It may be consumed directly, consumed and used to produce brood food or royal jelly, or stored and consumed later. While larval bees may consume small quantities of raw pollen directly, they as well as the queen depend on processed secretions (brood food and royal jelly) produced by nurse bees. Availability and quality of pollen can have a great influence on the health status of the colony. Nectar is used as a source of carbohydrates, and may be consumed directly or stored inside the hive and converted to honey.

Honey bees typically forage in the middle of the day for food within 1-2 miles (2 - 3 km) of the hive, but may forage 5 miles (7 km) or more if high quality food is lacking nearby. From a risk assessment perspective, the large forage area of honey bees complicates the task of estimating potential exposure, as they may come into contact with multiple pesticides. The time of day when foraging occurs in relation to pesticide application also complicates risk assessment and risk management. Numerous other factors should be considered in light of bee biology which can impact the design or interpretation of data intended to inform pesticide risk assessment with these organisms.

CHAPTER 3 OVERVIEW OF NON-*APIS* BEES

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Introduction

While globally, honey bees (*Apis mellifera* L.) are used in pesticide toxicity testing to represent all non-target pollinating insects, there are also some intrinsic difficulties in using *Apis mellifera* for toxicity testing of pesticides. For example, field tests are challenging because they have a very long foraging range — median foraging distance of up to 6.1 km (covering 132 km^2) and maximum beyond 9.5 km (covering 283 km^2) (Beekman & Ratnieks 2000) — and the day-to-day variability of their foraging area and floral resources they visit (Visscher & Seeley 1982). In semi-field tests, honey bees do not respond well to being kept in cages or indoor environments.

In addition, there are many uncertainties regarding the extent to which pesticide toxicity data for honey bees can be considered adequate for assessing risk to other pollinator species, the majority of which are non-*Apis* bees. Studies have demonstrated variable and inconsistent toxicity among various bee groups (Torchio 1973, Johansen et al. 1983, Malaspina & Stort 1983, Macieira & Hebling-Beraldo 1989, Peach et al. 1994, Malone et al. 2000, Moraes et al. 2000, Scott-Dupree et al. 2009, Roessink et al. 2011). This variability results, in part, from the basic biological differences between the highly social honey bees (where a whole colony in many ways acts as a single biological unit), social bumble bees (*Bombus* spp.), stingless bees (Meliponini), and the mostly solitary (non-social) other bees, as well as the differences in physiology, life cycle, and behavior between any two insect species (Thompson and Hunt 1999).

The need to thoroughly explore hazard tests for non-*Apis* pollinators is more important now than in the past because many areas around the world are seeing increasing demand for insect pollination, but decreasing availability and rising costs for honey bee colonies to satisfy the needs of agriculture (Aizen and Harder 2009). As a result, across the globe many farmers are looking to other managed or wild (unmanaged) non-*Apis* bee species, and scientists are documenting that many crops are pollinated to a significant level by non-*Apis* bees. For example, managed bumble bees (*Bombus* spp.) are increasingly being used to support agricultural/horticultural production. Over 1 million bumble bee colonies of different species were sold worldwide in 2006, primarily for greenhouse fruit and vegetable production (e.g., tomato *Lycopersicon esculentum*), but also increasingly for commercial orchards and seed production (Velthuis & Doorn 2006).

In the U.S., many growers of alfalfa seed (*Medicago sativa*), almond (*Prunus dulcis*), apple (*Malus domestica*), blueberry (*Vaccinium* spp.), and sweet cherry (*Prunus avium*) are using managed solitary bees such as wood-nesting alfalfa leafcutting bees (*Megachile rotundata*) and blue orchard bees (*Osmia lignaria*), and ground-nesting alkali bees (*Nomia melanderi*). In some places, the use of these non-*Apis* pollinators is already widespread or is becoming more common (Bosch and Kemp 2001). For example, in the U.S. approximately 35,000 tons of alfalfa seed are produced annually (Pitts-Singer 2008) and in 2009 growers are estimated to have paid as much as $18.5 million (217,000 gallons at $85 per gallon; 821,434 liters at $22.45 per liter) to purchase alfalfa leafcutting bees from Canada (Stephen 2003, Mayer and Johansen 2003, James 2011, Pitts-Singer pers. comm. Dec 9, 2011). In Japan, the hornfaced bee (*Osmia cornifrons*) is managed to pollinate orchards of apple and pear (*Pyrus communis*) (Matsumoto et al. 2009), and in Brazil, the carpenter bee *Xylocopa frontalis* can be managed to pollinate the passion fruit (*Passiflora edulis*; Freitas & Oliveira Filho 2003). In Kenya, solitary bees have not yet been commercialized for pollination purposes, but efforts are underway to develop management protocols for solitary bees such as *Xylocopa calens*, *X. incostans*, and *X. flavorufa* for high-value greenhouse crops (Kasina, pers. comm. Oct 5, 2011).

In the tropics, efforts are also underway to develop meliponiculture (stingless bee keeping) as a source of revenue from honey production, other hive products, and rentals for crop pollination. Meliponiculture is well established in countries such as Brazil and Mexico (Nogueira-Neto 1997, Villanueva-Gutiérrez et al. 2005). In Africa there are ongoing efforts to improve the management and expand the use of regionally native stingless bees, for example in Ghana (Kwapong et al. 2010) and in Kenya (Kasina pers. comm. 2011).

At the same time, across the world, there is a growing emphasis on the role of unmanaged or wild bees in agro-ecosystems among agriculture and conservation agencies. For example, in the U.S. this includes national-level ecosystem restoration efforts by the U.S. Department of Agriculture's Natural Resources Conservation Service (USDA-NRCS), mandated under the *Food, Conservation and Energy Act of 2008* (Vaughan and Skinner 2009). These conservation efforts are based upon general trends demonstrating declines in populations of wild bees in agricultural landscapes (Kremen et al. 2004, Biesmeijer et al. 2006, National Research Council 2007), as well as the increasingly large body of research demonstrating the significant role that unmanaged non-*Apis* bees may play in crop pollination (Kremen et al. 2002, Kremen et al. 2004, Njoroge et al. 2004, Winfree et al. 2007, Campos 2008, Winfree et al. 2008, Kasina et al. 2009, Isaacs & Kirk 2010, Vieira et al. 2010, Carvalheiro et al. 2011). Furthermore, recent research highlights the importance of a diverse pollinator guild for optimum pollination (Klein et al. 2003, Höhn et al. 2008), as well as the benefits of the interaction between honey bees and wild bees to enhance the pollination effectiveness of honey bees (Greenleaf and Kremen 2006, Carvalheiro et al. 2011).

Non-*Apis* bees are often specialized for foraging for pollen on particular flower taxa, such as squash, berries, forage legumes, or orchard crops (e.g. Tepedino 1981, Bosch and Kemp 2001, Javorek et al. 2002, Brunet and Stewart 2010). This specialization is usually associated with more efficient pollination on an individual bee visit basis, which can lead to production of larger and more abundant fruit or seed from certain crops (Greenleaf and Kremen 2006, Klein et al. 2007, but see also Rader et al. 2009). In one study, researchers estimated that native bees contribute an estimated US$3 billion worth of crop pollination annually to the U.S. economy (Losey and Vaughan 2006). More recently, researchers estimated that in California alone, unmanaged non-*Apis* bees pollinated US$937 million to US$2.4 billion worth of crops (Chaplin-Kramer et al. 2011).

In addition to their impact on agroecosystems, non-*Apis* pollinators are crucial to the native flora. More than 85% of flowering plants benefit from animal pollinators (Ollerton et al. 2011), most of which are insects and the most important of which are bees (Apiformes). To develop appropriate toxicity tests and risk assessment protocols for non-*Apis* bees, however, it is important to understand more about non-*Apis* bees and the unique exposure pathways relevant for them.

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Because of this increase in our understanding of the value of non-*Apis* bees for agriculture, and the critical role they play in natural ecosystems, researchers have voiced concern that the current pesticide risk assessment's focus on western honey bees as a surrogate pollinator species may not provide sufficient protection. They have recommended that testing include at least one solitary managed species, such as the wood-nesting alfalfa leafcutting bees (*Megachile rotundata*) or the blue orchard bees (*Osmia lignaria*) (Abbott et al. 2008, Ladurner et al. 2008), and one managed social non-*Apis* bee, such as bumble bees (e.g.,

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Bombus impatiens or *B. terrestris*) in temperate climates (Thompson and Hunt 1999) and/or the highly social stingless bees (e.g., *Melipona* spp. or *Trigona* spp.) in the tropics (Valdovinos-Núñez et al. 2009).

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To best develop appropriate toxicity tests and risk assessment protocols for non-*Apis* bees, however, it is important to understand more about the differences in biology between *Apis* and non-*Apis* bees, and the differences in exposure pathways. It is also important to recognize that these differences may also provide opportunities for improving the study design of toxicity tests that currently rely solely on honey bees.

Non-Apis Bee Biology and Diversity

Worldwide, there are over 20,000, recorded species of bees (Michener 2007, Ascher & Pickering 2011). They range in size from approximately 2 mm (1/12 inch) to more than 25 mm (1 inch), exhibit a wide variety of foraging and nesting strategies, vary from solitary to highly social, and exhibit other diverse life histories.

Bees use nectar mainly as a carbohydrate source and pollen as a source of protein, fatty acids, minerals, and vitamins. Some species also use other plant resources such as resins, leaves, plant hairs, oil, and fragrances to feed their larvae, build and protect nests, or attract mates (Michener 2007). Because they use plant products during all life cycle stages, they are vulnerable to plant protection products that are present or expressed in pollen and nectar, or that are found in or on other plant resources.

During their life cycle, bees undergo a complete metamorphosis where they develop through egg, larval, pupal, and adult stages. It is only the last of these, the adult, which we see and recognize as a bee. During the first three stages, the bee is inside a brood cell of the nest. The length of each stage varies widely between species, and is often defined by whether the bee is solitary or social (O'Toole and Raw 1999). The majority of species are solitary; each female works alone to create a brood cell, place a mixture of pollen and nectar into it, and then lay an egg on (or more rarely in) the food. Solitary bees may take a year to complete metamorphosis, although it can happen faster — 4 to 6 weeks — in those species that have 2 or 3 generations per year. Social bees, on the other hand, take only a few weeks to complete growth and emerge as adults.

The quantity of food provided at the time of egg-laying depends on whether the larvae is mass-provisioned (i.e., all of the bee's food is supplied in the cell at one time), or if the larvae is progressively fed (i.e., the food is delivered in small doses over time). Most solitary bees mass-provision their brood cells, as do most stingless bees, whereas honey bees and most bumble bees feed their brood progressively.

Female bees of most species have special morphological structures that enable them to carry pollen back to their nests. For example, the tibiae on the hind legs of honey bees, bumble bees, and stingless bees are modified into corbiculae (a flattened, shallowly depressed area margined with a narrow band of stiff hairs) into which the bee accumulates pollen wetted with nectar and packed into place. Other bee species have scopae to transport pollen. Scopae are fringes, tufts, or brushes of hair on their legs, their thorax, or the undersurface of the abdomen. Scopae are used to transport large amounts of pollen, usually in a dry state.

The wide range of different life history traits of bees has implications for their exposure to pesticides (Brittain and Potts 2011) and so below is describe relevant aspects of their natural history.

Generalist and Specialist Foragers

Bee species have two primary strategies for collecting pollen: (1) generalist (polylectic) foragers, such as honey bees (*Apis* spp.), stingless bees (Meliponini), and bumble bees (*Bombus* spp.), that gather pollen from a wide range of flower species and (2) specialist (oligolectic) foragers that gather pollen from a narrow range of plant species that are usually related taxonomically. Examples of oligolectic bees are squash bees (*Xenoglossa* or *Peponapis* spp.), *Macropis* spp., and *Leioproctus* spp. which collect pollen from cucurbits (*Cucurbita* spp.), yellow loosestrife (*Lysimachia* spp.), and geebungs (*Persoonia* spp.), respectively. Very few bees are monolectic, where they feed on pollen from only a single species of plant. Oligolectic bees may gather their nectar from a greater range of flower species than those they visit for pollen. The life cycle of specialist bee species is normally closely tied to their host plants, with the adult female bees emerging from their brood cells when their main pollen sources are flowering (O'Toole and Raw 1999).

Social and Solitary Behavior

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Bees exhibit a wide range of social behaviors, but can be broadly divided into two groups, social or solitary, depending on the interdependency individuals have with each other [Note:

there is a full range of social behaviors that occur between these two extremes]. Social bees live as a colony in a nest with one queen (or occasionally more). The labor of building the nest, caring for offspring, protecting the colony, and foraging for resources is shared amongst sterile female offspring of the queen.

Only a few species of bees demonstrate highly social (eusocial) behavior. These eusocial species include all the species of honey bees in the genus *Apis*, and approximately 400 stingless bee species in the tribe Meliponini (e.g., the genera *Trigona* or *Melipona*). Eusocial bees are found primarily in the tropics and subtropics, with two species, *Apis mellifera* and *Apis cerana*, living in temperate areas. Primitively social (or facultatively eusocial) bumble bees (genus *Bombus*) and some sweat bees (e.g., a subset of species in the genus *Lasioglossum*) exhibit lesser degrees of eusocial behavior (Michener 2007), where colonies are initiated by queens or dominant females on an annual basis. Most remaining bee species — the vast majority — are solitary. For these solitary species, the labor of nest construction and provisioning, foraging and egg-laying is all done by single, fertile female bees. Although solitary bees sometimes will nest together in great numbers, these gregarious bees are not cooperating (Michener 2007, Cane 2008).

In the world's temperate zones, bumble bees are the best known non-*Apis* social bees. Bumble bees live in colonies, share the work of foraging and nest construction, and produce many overlapping generations throughout the year, thus they are eusocial. However, unlike honey bees, bumble bee colonies are seasonal. At the end of the summer, most of the bees in the colony die, leaving only a few fertilized queens to hibernate (usually underground) through the winter. In the spring, each surviving queen will start a new nest, which may eventually grow to include dozens to hundreds of workers, depending on the species. Apart from honey bees, bumble bees are often the first bees active in late winter (foraging at lower temperatures than honey bees) and the last bees active in the autumn (Kearns and Thomson 2001, Goulson 2003).

Most bumble bees are generalist foragers, visiting a wide diversity of flowers. Bumble bees can gather pollen by "buzzing" flowers — holding them tightly and vibrating their flight muscles (with an audible buzz) causing the poricidal anthers to release their pollen. Buzz pollinators are important for ensuring pollination in crops with poricidal anthers such as blueberries, cranberries, and other *Vaccinium* spp., as well as solanaceous plants including tomatoes and eggplants (*Solanum melongena*), but also others such as peppers (*Capsicum annuum*) and strawberry (*Fragaria x ananassa*).

Bumble bees need a suitable cavity in which to nest. Sometimes they build nests above ground, under a tussock of grass or in hollow trees or walls, but generally they nest underground (Kearns and Thomson 2001). Abandoned rodent burrows are common nest sites, as this space is easily warmed and likely contains nesting and insulating materials, such as fur or dried grass. In this cavity, the queen creates the first few pot-like brood cells from wax secreted by her wax glands, lays eggs, and then forages to provide her brood with pollen and nectar (Goulson 2003). It will take about a month for her to raise this first brood. When this first brood emerges, these bees become workers. They take on the task of foraging and help the queen tend the growing number of brood cells through the summer. At the end of summer, new queens and drones emerge and mate. When the cooler weather of fall arrives most of the bees, including the old queen, will die, leaving only the new, mated queens to find appropriate sites in which to hibernate through the winter (Kearns and Thomson 2001).

Bumble bees mainly occur in temperate areas. However, as the pollination demand for greenhouse crops grows in the tropics, there have been attempts to introduce bumble bee colonies in these countries. The threats of such introduction may include inbreeding with local bumble bee species, competition with the native bees for food resources, and transfer of pathogens (Oldroyd 1999, Thomson 2004, Stout and Morales 2009), which may result in a decline in the abundance and/or diversity of the native bee community (Dafni et al. 2010) and disruption to the pollination of native plants. In temperate countries, the approach of winter checks the population of these bees through the death of all caste members except newly mated queens. In warmer climates, weather may be more favorable all year round and these bees may not diapause, increasing their numbers tremendously within a short duration of their introduction (Beekman et al. 1999, Dafni et al. 2010). Thus, there is a need to study locally or regionally native stingless bees to provide pollination service for greenhouse crops in the tropics (Slaa et al. 2000, Del Sarto et al. 2005).

Stingless bees live in the tropical and southern subtropical areas (Michener, 2007). They range in size from 1.8-13.5 mm in length and are sparsely to moderately hairy. They live in colonies that number from a few dozen individuals to more than 25,000, and they are active year-round. The colony size and nest architecture are characteristic for each different species. The greatest number of species is found in Central and South America where they have been

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domesticated since well before the arrival of Columbus. In the Yucatan Peninsula, farming of stingless bees for honey and wax was so extensive that European honey bees were not introduced until the 19th century (Crane 1992, Vit et al. 1994, Javier et al. 2001).

Stingless bees are generalist foragers, visiting a broad variety of flowers. However, individual colonies or populations may demonstrate a tendency to visit particular types of flowers or exhibit a temporary fidelity to specific plant species (Ramalho et al. 1994, 1998, 2007). They are known to visit at least 90 crop species and are used to enhance pollination in some crops on a commercial to semi-commercial basis (Heard & Dollin 1998a, Heard 1999).

Most stingless bees nest in a cavity. Typically, these cavities are in trees or hollow logs; however, a few species will move into termite mounds, building walls, or even cavities underground. Nests are often located 2 to 30 m above ground (Kajobe 2007). Stingless bees line their nest cavity with an envelope of batuman, a tough mixture of wax produced by the bees combined with resins, gums, plant material, and sometimes mud collected from around the nest. The nests are composed of many storage pots of honey and pollen, and smaller brood cells. The pots (both storage and brood) are made of cerumen, a mixture of wax and plant resins.

Within the nest, each brood pot is mass provisioned with hypopharyngeal gland secretions, pollen, and honey. An egg is laid on top of these provisions and then the pot is sealed. The nests can have one to several queens depending on the species. Most species of stingless bees have brood cells of two different sizes; the large cells produce gynes (queens) while the small ones produce males and workers (Michener 1974). Caste determination is usually through food provisioning, with the quantity, not the quality, of food determining the caste. Thus gyne cells are provisioned with more food compared to the worker and male brood cells. This is in contrast to the honey bee caste determination where both quantity and quality of brood food is important.

New nests are initiated on a progressive basis. A virgin queen moves into a new cavity with some workers over a period of some weeks. They take materials from the old nest to create the new. Hence stingless bees are not capable of long distance migration (Roubik 2006). However, with domestication, new colonies can be established through methods similar to splitting honey bee colonies. Young gynes are moved together with brood, workers, and males to a new hive to establish a new colony (Nogueria-Neto 1997, Arzaluz et al. 2002, Villanueva-Gutiérrez et al. 2005, Kwapong et al. 2010).

Solitary bees

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The majority of bee species in the world are solitary. A female solitary bee may lay twenty or thirty eggs in her life. For solitary species having one generation per year, one to three weeks after an egg is laid, it hatches and larva emerges to feed on the pollen and nectar ("bee bread") previously provided by the adult female. The larva grows rapidly for six to eight weeks before pupating. The dormant prepupal or pupal stage typically lasts eight or nine months in temperate climates. When it emerges, the adult bee is fully grown and then needs food (primarily nectar) for egg maturation and energy. Most solitary bees have only one generation per year, and have a fairly short season of adult activity. Some solitary species, such as some sweat bees in the genera *Halictus* and *Lasioglossum*, have two or three generations each year and so are present over a long period of time.

Adult solitary bees are typically active for three to six weeks. Males usually emerge first from the nest, after which they typically loiter around a nesting area or a foraging site in search of a female to mate with. After a female bee emerges, she mates and then spends her time building and provisioning a nest in which to lay eggs (O'Toole and Raw 1999, Michener 2007, Cane 2008). The adults of a species emerge at roughly the same time each year: for example, early spring in the case of blue orchard bees (*Osmia lignaria*) or midsummer in the case of squash bees (*Peponapis pruinosa*). This emergence normally coincides with the flowering of forage plants, particularly if the bee is a specialist.

About 30% of solitary bee species are twig or wood-nesting. Most species use hollow stems or abandoned beetle burrows or other tunnels in dead or dying standing trees, but some can chew out a nesting tunnel in the soft central pith of stems and twigs, or – in a few cases – they may bore their own tunnel in wood (Michener 2007). The other 70% nest in the ground, digging tunnels in bare or partially vegetated, well-drained soil (Potts et al. 2005). Each solitary bee nest will have one or more separate cells in which the female places all the provisions (pollen and nectar) required for the full development of her larva. While some nests may have only a single cell, most have five or more. In the case of ground-nesting bees, females create a range of underground architectures, from simple tunnels to complex, branching systems with cells usually located 10 cm to 2 m underground. Wood-nesting bees on the other hand, usually stack cells in a single line inside their nest tunnels.

Most wood-nesting species separate individual brood cells with materials they collect, such as leaf pieces, leaf pulp, plant hairs, tree resin, or mud. For example, leafcutting bees (genus *Megachile*) use pieces of leaf or petal to create self-contained brood cells. Using their mandibles, they cut particular sizes and shapes to fit different parts of the brood cell, lining the entire cell. Most other wood-nesting bees, however, do not line the entire cell, but simply build dividing walls across the nesting tunnel, segmenting it into separate brood cells. Blue orchard bees (genus *Osmia*) make these walls with mud or leaf pulp. Large carpenter bees (genus *Xylocopa*) and small carpenter bees (genus *Ceratina*) use wood fibers scraped from the walls of the tunnel to form dividers of compacted sawdust. These bees seal the nest entrance when it is finished with the same materials they used to construct the inner partitions.

Rather than collecting materials from outside the nest with which to line their brood cells, many ground-nesting bee species smooth the cell walls with their abdomens and then apply a waxy or oily substance produced from special glands near their mouths or on their abdomens to line the cells, thus stabilizing the soil and protecting their brood. The substance lining the cell usually soaks into the soil, making it look shiny and helping to exclude water and control microbes. Plasterer or polyester bees (genus *Colletes*), yellow-faced bees (genus *Hylaeus*), and other bees from the family Colletidae line each cell with a cellophane-like substance secreted from special glands to create a complete waterproof lining for their underground cells. A few species, such as tiny *Perdita* bees living in the southwestern deserts of the United States, leave their underground cells unlined.

Status of Toxicity Testing for Non-Apis Bees

In general, the research on pesticide toxicity and risk assessment for non-*Apis* bees lags far behind that for honey bees (see Table 1 for examples of pesticide toxicity studies conducted on non-*Apis* bees). Except for bumble bees, most of the data referred to on non-*Apis* bees has been sourced from North America. The most commonly studied species are *Megachile rotundata* (the alfalfa leafcutting bee), *Bombus impatiens* (the eastern bumble bee), and *Osmia lignaria* (the blue orchard bee), all of which are managed species of economic importance. These species have been put through a range of lower and higher tier toxicity

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tests, but only for a handful of active ingredients, usually of regional importance. At present, the tests are not standardized.

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Most of the non-*Apis* bee toxicity testing conducted in Europe has been on bumble bees, and in particular *Bombus terrestris*, which is the main species used for commercial pollination. Typically, bumble bee suppliers (e.g., Koppert Biological Systems, Biobest, and Syngenta Bioline) complete thorough higher tier testing of pesticide toxicity to ensure bumble bee safety in greenhouses when pesticides have to be applied. Lower tier toxicity tests (e.g. acute toxicity tests conducted in the laboratory) are somewhat limited, but comparative toxicities between *A. mellifera* and *Bombus* spp. have been reviewed by several authors (Thompson 2001, van der Steen et al. 2008). Comparison has been made both on a dose per bee level and a dose per gram of bee (factoring in the larger size of the bumble bee). The broad conclusions are that there is no consistent correlation between the toxicity for *Apis* and *Bombus* workers, but the general trends suggest that the toxicity to bumble bees is less on a per bee basis and similar on a per gram of bee basis.

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Work on the comparative toxicity of pesticides to individual/colonies of stingless bees in the subtropics and tropics is in its relative infancy. In part, this is because little is known of the biology of most stingless bee species and many species remain undiscovered or undescribed. However, because there is significant interest in the management in these species for the pollination of high value crops, the need to understand the effects of pesticides is growing. Already some toxicity work has been done using various species of Meliponini (*Melipona beecheii*, *Trigona nigra* and *Nannotrigona perilampoides*; Valdovinos-Núñez et al. 2009). Collaborations are underway between national regulatory authorities, national research institutions, and universities to develop toxicity testing protocols for non-*Apis* bees commonly used for field or greenhouse pollination in the tropics. Using OECD guidelines (OECD 1998) as a template protocol, these toxicity tests are being developed by partners in Brazil, Kenya, and the Netherlands to carry out comparative studies with native stingless bees, solitary bees, honey bees, and bumble bees (Roessink et al. 2011). Specifically, stingless bees in Kenya currently being studied include *Meliponula ferruginea* and *M. bocandei*, while in Brazil they include *Scaptotrigona postica* and *Melipona scutellaris*. The African honey bee (*Apis mellifera scutellata*) in Kenya and the Africanized honey bee (also *Apis mellifera scutellata*, but hybridized with European honey bees in the Americas) in Brazil are also study organisms. The results are expected to aid in understanding differences in sensitivity to various pesticides among stingless bees and honey bees in the tropics,

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compared to the western honey bee (*Apis mellifera mellifera*) and bumble bee (*Bombus terrestris*) found in the Netherlands. In addition, tests will be performed on solitary bees in Brazil and Kenya (e.g., *Xylocopa* spp.) after optimizing procedures for their rearing to ensure enough individuals are available to meet the testing requirements.

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Opportunities for non-*Apis* bees to inform pollinator risk assessment

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Some of the life history traits of non-*Apis* bees described above lend themselves to providing very useful information for risk assessors. For example, when tiered assessment protocols lead to field testing of a pesticide, it is usually only feasible to apply the product to a small area (e.g., ≤ 2 ha.) of a bee-attractive crop, and then place honey bee colonies within or adjacent to that crop. Those colonies will routinely forage over more than a thousand hectares (Visscher & Seeley 1982, Steffan-Dewenter & Kuhn 2003), and it is difficult to control what the bees from these colonies actually forage on and therefore to what extent they encounter the product being tested. Even when hives are fitted with pollen traps to check that some foragers are visiting the focal crop, it is not certain that they are actually foraging on the treated area or in other fields of the same crop in their foraging range. Furthermore, the dilution factor of the pollen and nectar harvested is usually considerable and varies significantly from one day to the next (Visscher & Seeley 1982). Solitary non-*Apis* bees, such as *Osmia* and *Megachile* spp., have a more restricted foraging area and scientists can typically be more confident that these bees are foraging on the treated crops (Maccagnani et al. 2003, Zurbuchen et al. 2010). Consequently, it is possible to gather more precise data on pesticide exposure ~~and effects~~ in the field, and extrapolate to potential impacts on bees when tens or hundreds of contiguous acres are treated in real-world situations.

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Non-*Apis* bees, especially managed species of social (i.e., bumble bees and stingless bees) and solitary bees, also lend themselves to semi-field experiments as they are much less stressed than honey bees in enclosed cage or greenhouse settings, and thus behave more "naturally." Table 2 provides a list of species that may be used for toxicity testing.

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Uncertainties in Current Test Designs for Non-*Apis* Bees

As we detailed above, there are many differences between the biology and life-history traits of *A. mellifera* and non-*Apis* bees. As a result, non-*Apis* bees have many unique exposure pathways or vulnerabilities to pesticide applications (Tuell & Isaacs 2010, Brittain & Potts 2011).

Larval Food Sources

Honey bee larvae are fed by adult workers (nurse bees) that provide royal jelly for the larva's first six days, after which larvae are fed a mix of royal jelly, pollen, and diluted honey. The majority of food for developing honey bee larvae comes from glandular secretions of workers (i.e., royal jelly), and these glandular secretions may result in modifications (e.g., degradation) of pesticide active ingredients in food stores. Indeed, direct pollen feeding by *Apis mellifera* larvae comprises less than 5% of the total protein consumed during larval development (Babendreier et al. 2004). As such, the and toxicity of pesticides to honey bees eating processed pollen/nectar (e.g., royal jelly) may differ from non-*Apis* bees, such as bumble bees and solitary bees – and likely stingless bees, as well – whose larvae feed directly on mostly unprocessed pollen, nectar, and other floral resources (O'toole & Raw 1996, Pereboom 2000). Thus, non-*Apis* larval exposure to pesticide-contaminated pollen and nectar is potentially much higher. With this in mind, exposure estimates based on stored honey bee pollen which is converted to royal jelly is unlikely to be predictive of the chemical residues fed to non-*Apis* bee brood (Konrad et al. 2008).

Egg and Larval Contact with Pollen Stores

In bees that mass provision their cells (i.e., most non-*Apis* bees), the egg and larvae are in direct contact with the pollen and nectar provision (bee bread). This larval food can be contaminated with systemic insecticides (Laurent & Rathahao 2003), and this contamination – when it occurs – is in direct contact with the most vulnerable life stages, i.e. the egg and 1^{st} larval instar. Honey bees, in contrast, are isolated in their cells and are fed progressively by nurse honey bees, hence have a very different exposure profile (Winston 1987).

Foraging Time and Duration

Pesticide applications in the evening and during periods of cool temperatures are sometimes recommended as ways to reduce bee mortality (Johansen & Mayer 1990, Tew 1997, Riedl et al. 2006), especially in locations experiencing cooler climatic conditions. These recommendations are based upon the premise that honey bees usually do not forage when temperatures are below 13°C (55°F) or between late evening and early morning (Johansen and Mayer 1990), thus giving pesticides with a short residual hazard more time to become inactive or less biologically available. However, this premise does not reflect the cooler weather tolerance of some temperate species of native bees, such as *Bombus* spp. and *Osmia* spp., both of which are frequently noted for their ability to forage during cool, inclement weather, as well as earlier and later in the day (Thompson and Hunt 1999, Bosch and Kemp 2001). Furthermore, the peak foraging times for bumble bees are very early and late in the day, whereas peak honey bee foraging typically occurs at different periods. Hence, mitigation recommendations such as applying pesticides early in the day may disproportionately affect bumble bees (Thompson 2001).

Similarly, squash bees (genus *Peponapis*) have been documented to perform a significant amount of pollination in the pre-dawn hours when honey bees are inactive (Sampson et al. 2007). Under such scenarios, recommendations to conduct night-time spraying – which is still preferable to spraying during the daytime – may result in disproportionately greater pesticide exposure to these key non-*Apis* pollinators. In particular, dewy nights may cause an insecticide to remain wet on the foliage and be more toxic to bees the following morning (Johansen & Mayer 1990, Tew 1997). In some instances, spraying crops that are soon to bloom (e.g., those at budburst) may have a disproportionately higher impact on male solitary bees that emerge before the females and often spend the night in flowers or attached to bud stems.

Nesting and Distance to Crops

Most non-*Apis* bees, especially soil-nesting species, cannot be relocated as a protection measure. [Notable exceptions include managed nests of *Bombus*, *Osmia*, and *Megachile* spp.] Many non-*Apis* bees will nest in the ground in orchards and even within row crops (Kim et al. 2006). Squash bees (genus *Peponapis*), for example, frequently nest underground at the base of squash and pumpkin plants within production fields (Shuler et al. 2005), as do *Melissodes* bees in cotton fields (Vaissière et al. 1985). Therefore, recommendations made to protect honey bees by closing up or moving hive boxes are of little value for economically important wild bees living in and around crop fields and orchards. Similarly, some alfalfa seed producers in western U.S. states rely on artificially constructed salt flats to aggregate large numbers of ground-nesting alkali bees (*Nomia melanderi*) for pollination (Cane 2008). The large size of such nesting areas, the long distance these bees can fly (up to 3.2 km [2 miles]), and their potential location away from seed production fields makes it impossible to close off nest entrances to prevent them from foraging in recently sprayed fields.

Nesting materials

Some non-*Apis* bees (e.g., *Megachile* spp.) use materials such as excised leaf or petal pieces to encase developing brood and brood provisions. Stingless bees build their nests with resins they collect from the environment. Several studies have identified pesticide contamination of these nest materials as a significant cause for concern, particularly in the case of pesticides with a long residual toxicity (Waller 1969, Johansen et al. 1983, Johansen and Mayer 1990). The increasing use of newer systemic insecticides, including those labeled for landscape use, may pose even greater threat of nest material contamination for leafcutting bees (Krischik, personal communication, 12/12/2011), but require further study to document the actual risk.

Size

Some non-*Apis* bees can be much smaller than honey bees (e.g. bees of the genera *Perdita* or *Dialictus* in the U.S. and *Nomioides* in Europe), and therefore receive a relatively higher dose because of the higher surface area to volume ratio of smaller bodies. Indeed, even intra-specific tests of pesticide toxicity to bumble bees have confirmed that smaller bees have a greater risk of mortality at lower doses (van der Steen 1994, Thompson and Hunt 1999, Malone et al. 2000).

A second size-related factor affecting hazard risk of pesticides to bees is the direct relationship between foraging distance and species size. While large bees, such as bumble bees or alkali bees, easily forage 1 km or more from their nest, small bees may only fly 200 m from their nest site (Greenleaf et al. 2007) and only the strongest and largest individuals have the capability to cover longer foraging distances (Zurbuchen et al. 2010). This factor potentially results in a disproportionate risk to small bees that are attracted to blooming crops, where their limited foraging range necessitates nearby nesting, and ongoing exposure to pesticide applications throughout the growing season. In some studied landscapes (e.g., New Jersey, USA), small bees (e.g., *Halictus* and *Lasioglossum* spp.) perform a significant amount of crop pollination (Winfree et al. 2008).

Forage Areas

Non-*Apis* bees may forage on plants that are seldom visited by, or do not require pollination from, honey bees (e.g., tomato, potato (*Solanum tuberosum*), many legumes, some ornamentals). For example, some solanaceous crops such as tomatoes or potatoes produce no nectar and have pollen in large anthers that open via two small pores. Honey bees typically do not visit these plants because of the lack of nectar and difficulty in accessing the pollen. However, some non-*Apis* species, such as bumble bees (*Bombus* spp.) and *Anthophora* spp., sonicate (buzz) these flowers, releasing large bursts of pollen by vibrating the anthers. As a result, insecticide applications to these crops may be considered safe for honey bees, but will potentially poison foraging non-*Apis* species.

Impact of field kills

When honey bee workers are killed in the field, the loss of these workers may, to a certain extent, be compensated by the colony, which may continue to grow and reproduce with little or no impact. Because most non-*Apis* bees are solitary species, where single female bees build their nests, lay eggs, and forage for pollen and nectar to feed their offspring, the death of a foraging female or even the incapacity to provision her cells results in the cessation of her reproduction (Taséi 2002). Field kills of bumble bee queens early in the season also has a disproportionately greater impact, as their death (as opposed to that of a worker) prevents a colony from being established.

Variety and dilution of pollen and nectar in social vs. solitary bees

Solitary bees will collect pollen repeatedly from one area, and often one or a few plant species, to bring back to the nest, whereas honey bees from a single colony are out foraging on a wide variety of plant species across a large landscape. Honey bee foraging areas and sources of nectar and pollen can vary considerably from one day to the next (Visscher & Seeley 1982). Thus, a toxin on one crop may be diluted in a honey bee colony foraging at various resources over time and space, but not for the progeny of a solitary bee foraging on a crop or area of habitat treated with a pesticide.

Conclusions

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It is clear that non-*Apis* bees play a critical role in supporting diverse plant communities, and an increasingly important role in agriculture. They face exposure routes from pesticides that do not occur for honey bees, and there are more limited risk management options available for their protection. Their intrinsic biological characteristics may make them generally more susceptible to pesticide effects than honey bees resulting in a greater impact from similar exposure. At the same time, these characteristics – such as their more limited foraging ranges and relatively unaffected foraging in enclosed areas – could be used to better assess the risks of pesticide applications for pollinators, including honey bees.

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Table 1. The availability of laboratory and field tests for representative groups of solitary and social non-*Apis* bees (see laboratory and field chapters for detailed protocols)

Study Type		**Solitary**		**Social**	
		Tunnel-nesting (tube, wood)	**Ground-nesting**	**Bombini (bumble bees)**	**Meliponini (stingless bees)**
Laboratory	**Adult**	Temperate north ***Megachile rotundata*** (Huntzinger *et al.* 2008; Scott-Dupree *et al.* 2009) ***Osmia lignaria*** (Ladurner *et al.* 2005; Scott-Dupree *et al.* 2009) Tests in development (tropics) ***Xylocopa* spp.**	Limited availability of tested species Temperate north ***Nomia melanderi*** (Johansen *et al.* 1984; Mayer *et al.* 1998)	Temperate north ***Bombus terrestris*** (for a review see Thompson 2001) ***Bombus impatiens*** (Scott-Dupree *et al.* 2009; Gradish *et al.* 2011b)[1]	Tests in development (tropics) **Several species in tropical western hemisphere** (Macieira & Hebling-Beraldo 1989; Valdovinos-Nunez *et al.* 2009)
	Larva	Temperate north ***Megachile rotundata*** (Peach *et al.* 1995; Gradish *et al.* 2011a, Hodgson *et al.* 2011) ***Osmia lignaria*** (Abbott *et al. 2008*) Tests in development (tropics) ***Xylocopa* spp.**	Not yet investigated	Temperate north ***Bombus terrestris*** (for a review see Thompson 2001) ***Bombus impatiens*** (Gradish *et al.* 2010; Gradish *et al.* 2011b)[1]	Tests in development (tropics)
Field	**Semi-field**	Temperate north ***Megachile rotundata*** (Johansen *et al.* 1984, Tasei *et al.* 1988, Mayer & Lunden 1999), ***Osmia bicornis*** (Konrad *et al.* 2008), ***Osmia lignaria*** (Ladurner *et al.* 2008)	Can be developed	Temperate north ***Bombus terrestris*** (Tasei *et al.* 2001) ***Bombus impatiens*** (Gels *et al.* 2002) (needs standardized guidelines)	Tests in development (tropics)

Field	Temperate north ***Megachile rotundata*** (Torchio 1983), ***Osmia lignaria***	Limited availability of tested species ***Nomia melanderi*** (Mayer *et al.* 1998)	Temperate north ***Bombus terrestris*** (Tasei *et al.* 2001), ***Bombus impatiens*** (needs standardized guidelines)	Tests in development (tropics)
Exposure Pollen, nectar, foliar, soil	Can be developed (for pollen provisions in the field see Abbott *et al.* 2008; for foliar resides see George & Rincker 1982)	Not yet investigated	Can be developed (for pollen see Morandin *et al.* 2005)	Can be developed

[1] Needs standardized guidelines of currently used lab bioassay and microcolony assays.

Table 2. Non-*Apis* bee species suitable for laboratory, semi-field or field tests. All of these species are either commercially available and/or they can be managed for crop pollination in various parts of the world. It is important to work with species that are appropriate for the region where research is conducted.

Species (common name)	Sociality	Region	References on management
Megachile rotundata (Alfalfa leafcutting)	Solitary	Temperate North America, Asia	Mader et al. 2010
Osmia lignaria (Blue orchard bee)	Solitary	Temperate North America	Bosch & Kemp 2001, Mader et al. 2010
Osmia cornifrons (Japanese orchard bee)	Solitary	Temperate Asia, Europe	Sekita & Yamada 1993, Wilson & Abel 1996, White et al. 2009, Mader et al. 2010
Osmia rufa (Red orchard bee)	Solitary	Temperate Europe	Krunic et al. 1995, Bilinski & Teper 2004
Osmia cornuta (Hornfaced bee)	Solitary	Southern and Central Europe	Krunic et al. 1995, Maccagnani et al. 2003
Amegilla chlorocyanea (Blue-banded bee)	Solitary	Australia	Hogendoorn et al. 2006
Xylocopa spp. (Carpenter bees)	Solitary	Tropical (Brazil)	Freitas & Oliveira-Filho 2001, Freitas 2004
Bombus impatiens (Eastern bumble bee)	Social	Temperate (North America)	Readily available commercially. See also Evans et al. 2007, Mader et al. 2010
Bombus terrestris (European bumble bee)	Social	Temperate (Europe)	Readily available commercially. See also Evans et al. 2007, Mader et al. 2010
Melipona beecheii (stingless bee)	Social	Tropical (Central America)	Gonzalez & De Araujo Freitas 2005, Villanueva-Gutiérrez et al. 2005, Quezada Euán 2005, Quezada Euán & José Javier 2009
Trigona nigra (stingless bee)	Social	Tropical (Central America)	González & Medellín 1991a, 1991b
Nannotrigona perilampoides (stingless bee)	Social	Tropical (Central America)	González & Medellín 1991a, 1991b
Trigona carbonaria (stingless bee)	Social	Tropical (Australia)	Heard 1998, Heard & Dollin 1998b, Greco et al. 2011
Melipona subnitida (stingless bee)	Social	Tropical (Brazil)	De Oliveira Cruz et al. 2005

Meliponini tribe (stingless bees)	Social	Tropical (Brazil)	Nogueira-Neto 1997
Trigonini tribe (stingless bees)	Social	Tropical (Brazil)	Nogueira-Neto 1997
Meliponula bocandei (stingless bee)	Social	Tropical (Africa, Kenya)	Kwapong et al. 2010
Meliponula ferruginea (stingless bee)	Social	Tropical (Africa, Kenya)	Kwapong et al. 2010

CHAPTER 4 OVERVIEW OF PROTECTION GOALS FOR POLLINATORS

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Introduction

The management of cropping systems has evolved over the past decades in a response to higher demands for food and fibre. Along with this came an increased need to control pest populations and diseases. Plant Protection Products (pesticides) are an integral part of commercial production. Protection authorities that regulate the use of pesticides serve a critical function in assessing and balancing the benefits of pesticides with other potential consequences of their use in order to maximize overall benefits to the societies they serve. Authorities articulate the overarching objectives of their efforts, in "protection goals" which serve as a guide and measure of their efforts.

Over time, entities such as the Organization for Economic Co-operation and Development (OECD), the US Environmental Protection Agency (EPA) and the European and Mediterranean Plant Protection Organisation (EPPO), have developed a number of documents to guide the risk assessment process which is a primary tool to support decision-making with respect to registering pesticides. However, variables other than estimated risk are also considered when making regulatory decisions, and may include economic, legal, or political considerations. Together, all the variables are considered and balanced in a way that produces a decision that is consistent with the protection goals of an authority.

A risk assessment process must be designed to provide clear information the risk assessor and risk manager to determine whether the proposed use of a pesticide product would, or would not be consistent with the protection goals of a regulatory authority. Therefore, participants of the Workshop spent some time discussing protection goals for pollinators such that a risk assessment process could be proposed that would serve the needs of regulators.

Elements and Proposed Protection Goals

Pollinators, and honey bees in particular, contribute significantly to the economy as an input to commercial agriculture, as well as through their production of hive products (e.g., honey, pollen, royal jelly, wax and propolis). Regulatory concern and interest in assessing the potential impact of pesticides to these organisms reflects a number of factors, among these are:

- the role of the organism plays in ecosystem services, such as in natural and cultivated systems
- the perception (*e.g.*, estimated exposure values) or knowledge (*e.g.*, monitoring data) of potential exposure of the species to plant protection products.
- Information on actual impacts of pesticides on pollinators (*e.g.*, incident reports or survey efforts).

In addition to the direct market value (with crops that require animal pollination, or hive products), investigations in the recent past have provided evidence of the relationship between declining pollinating species in cropped areas and reductions in crop yield (Kevan, 1999, Kluser *et al.*, 2007). Concerns that the use of pesticide products may have adverse impacts on honey bee health have led to the implementation of surveys and monitoring activities over the past several years in different parts of the world. Surveys in Europe have focused on recording and explaining incidents of declines in several European countries (Neuman et al, 2010), or in the US (van Englesdorp et al., 2010). By comparison, surveys have seldom been undertaken with the aim of describing the pollinating fauna from an ecological perspective (Kluser et al., 2007). Most surveys however, are implemented at the macroscopic level so that the outcome may only provide an alert of the occurrence of side-effects of cropping practices (including pesticides). In comparison, incident reports are a field-level indication of effects related to a particular product, or crop scenario. An example of this is the case that occurred in Germany in 2008 following the sowing of pesticide treated seeds. A detailed analysis of the conditions of occurrence of this beekill incident has been performed, the results of which have served as an "alert" on the possible risks that may result from an exposure to seed dusts at sowing, under certain circumstances (Foster, 2010). Finally, our knowledge of the potential impacts of pesticides on pollinating species is linked to the tools that are available to assess and characterize (quantitatively and qualitatively) any potential adverse effects. The honey bee, in this respect, is treated somewhat differently from other pollinating species and other taxa for which ecological risk assessments are conducted. In Europe for example, the honey bee is the only organism for which a dedicated risk assessment is performed at the species level. As a consequence, the knowledge of the possible impact that pesticides may exert on the honey bee is far more detailed and documented than for other pollinating species.

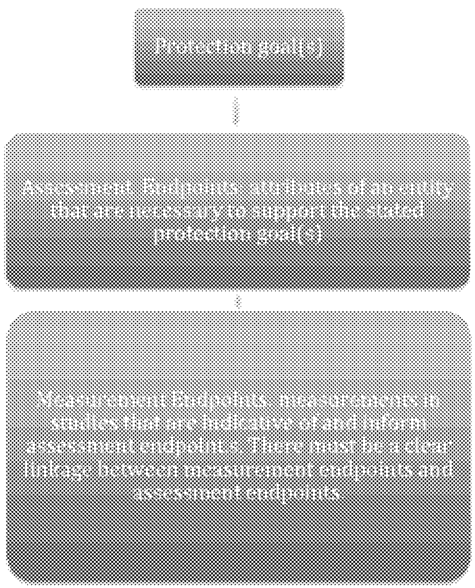
Protection goals therefore, reflect a certain level of information, and certain values of a society. Protection goals may be broad (*e.g.*, a healthy environment or productive agriculture, *etc.*) or narrow (amelioration of a water body); but, must be clearly articulated and must be transparent with respect to the importance of that goal to the man-made and/or the natural environment. As stated above, protection authorities use risk assessment tools to estimate potential adverse effects of pesticides to human health or the environment. Therefore, if a government or protection authority wishes to include pollinators within its protection goals, then risk assessment tools appropriate to assess potential risks are needed.

The participants came to the workshop with an understanding of the value of honey bees, and of the current science on potential exposure and effects of pesticides on bees. From this discussion developed surrogate protection goals that served the participants as they developed recommendations on a risk assessment process as well as the data that informs that process. However, the participants of the Workshop were aware that since protection goals do reflect values (including legal, and resource considerations) that are specific to a government or authority, it was not within the purview of the Workshop to define the protection goal of any one country or protection authority.

Participants of the Workshop agreed that a critical ecological service of pollinators (bees in particular) to be protected is maintaining the pollinating function of these organisms. The aim would be to ensure sufficient pollination (sufficient frequency of floral visits) to support healthy crop survival and yield. The corresponding protection goal would be defined as "maintining pollination services through the presence of sufficient bees to ensure crop production." Such a protection goal is relevant for agricultural production crop(s) of concern. However, such a definition may not be relevant at a larger scale, *i.e.* the [cropped] landscape, as it does not account for the role of non-*Apis* species that may serve in pollination of adjacent cropland or serve in the pollination of the non-cropped landscape. For this to be taken into account, non-*Apis* (i.e., non-managed) pollinating species would need to be considered with their interactions in the larger landscape. Regardless, pollination remains the actual function to ensure a healthy and ecologically diverse landscape. Protection of the pollinator community at the landscape level not only is the function of maintaining pollination services, but also includes ensuring the diversity of the species associated with pollination services within the landscape as a whole.

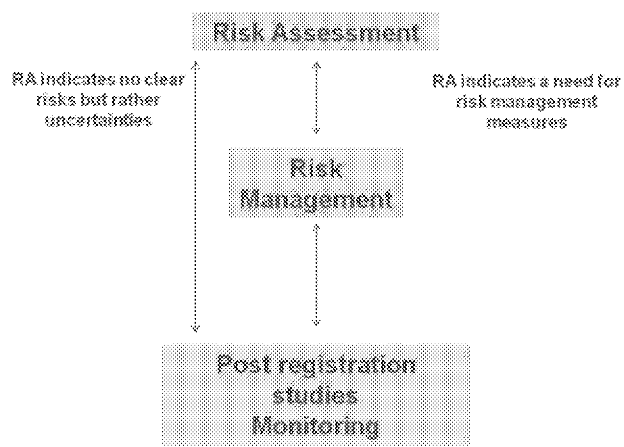
Having defined a possible protection goal, it then must be linked to risk assessment endpoints. Assessment endpoints are attributes of a entity (e.g., an organism or environmental component) that are essential for its continued survival. In ecological risk assessments for wildlife, assessment endpoints have traditionally been defined as the growth, reproduction and survival of an organism. We can apply these same assessments to the honey bee, but must be aware that the honey bee functions as a superorganism and therefore it is the growth, reproduction and survival of the colony, not the individual, that is relevant.

The risk assessment is based upon data that, collectively, indicate whether the assessment endpoints of growth, reproduction, and survival are maintained. Exposure studies and effects studies produce measurement endpoints (e.g., brood size, body length, or mortality). Therefore, there is vertical integration between measurement endpoints (at the test level), assessment endpoints (at the decision level), and protection goals (at the societal level). Below, Figure X diagrams the relationship between measurement endpoints, assessment endpoints and protection goals.



Protection Goals and Monitoring

Protecting both ecosystem services and the market value of organisms is essential and the risk assessment process for pollinators, as proposed by the participants of the Workshop is designed to support the protection goals that were articulated at the Workshop, and provides an avenue for feedback information to continue to inform the basis for concern and protection. Feedback information, such as incident or monitoring data, provides direct information on whether the regulatory decisions are effective, and whether protection goals are being achieved. However, field monitoring can be complex since field often reflects natural events/scenarios, such as disease, predation and competition. Because of this, it is important that when defining protection goals, consideration is given to the risk assessment parameters and potential monitoring parameters such that a transparent relationship between these two exist. The relationship between risk assessment, risk management techniques, field surveys and post registration studies may be illustrated as in Figure X.



A well defined protection goal guides a risk assessment by providing criteria for decisions within the paradigm of risk assessment (study design and interpretation), risk management, or post-registration monitoring actions. Protection goals must be reachable and sustainable through appropriate scientific analysis and decisions, (*i.e.*, studies, management, and/or monitoring). Both risk assessment and risk management are complementary options to meet protection goals. During the Workshop, participants discussed the long standing global importance of *Apis* and non-*Apis* bees in terms of both commercial and ecological realms. Participants then developed model (or surrogate) protection goals suitable upon which to build a risk assessment framework and defined them as:

(i) protection of pollination services provided by *Apis* and non-*Apis* species'

(ii) protection of honey production and other hive products; and,

(iii) protection of pollinator biodiversity, that is, protection of adequate number and diversity of bee species that contribute to the health of the environment (primarily non-*Apis* bees).

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CHAPTER 5 OVERVIEW OF THE PESTICIDE RISK ASSESSMENT AND THE REGULATORY PROCESS

As discussed earlier, regulatory authorities have the responsibility to evaluate plant protection products, (PPP, also known as pesticides), and the potential risks associated with their use; and, have developed tools and methods to do this with respect to different taxa. However, with the introduction of new plant protection products, changes in agricultural practices, and advances in the understanding of honey bee health and ecology, the ability to accurately characterize potential risks to insect pollinators with the existing tool set, has been seen as a challenge. While many countries share the same broad risk-based environmental assessment approach, differences between approaches exist that account for national conditions, such as policies, legal requirements, or preferences.

The Workshop considered a generic, tiered risk assessment methodology, and worked to develop a process that included three phases: problem formulation, exposure and effects assessment, risk characterization. In Phase 1, *i.e.*, *problem formulation*, measurement endpoints, derived from studies, are selected with an understanding of how they relate to assessment endpoints (and ultimately protection goals); a conceptual model is prepared that describes a risk hypothesis; and an analysis plan to test that hypothesis is described. In Phase 2, *i.e.*, *analysis*, measures of exposure and effects are evaluated. In Phase 3, i.e., *risk characterization* measures of exposure, and measures of effect are integrated to develop risk estimates, and uncertainties are discussed.

Analysis is done in a tiered manor, where a tier 1 analysis is intended to be a conservative screen that efficiently separates compounds that are not anticipated to present a potential risk from those compounds that may. Higher tiers are intended to refine the estimates or measures of potential exposure, potential effects, and the resulting characterization of risk. Assessors and managers proceed through the risk assessment process (*i.e.,* ascending through higher tiers of analysis) to determine whether the intended use of a compound is consistent with protection goals. If the estimate of risk indicates that proposed use is not consistent with protection goals, then risk mitigation techniques may be implemented proactively to resolve concerns. The Workshop did not directly address risk management, since it is technically outside the realm of assessment. However, it was briefly discussed as it is a component of the overall regulatory management of plant protection products. (see Chapter X on Risk Mitigation).

Current Approach for Assessing Effects of Pesticide Products to Pollinators

In the United States, the first tier of testing consists of an acute contact toxicity test[3] with adult honey bees that provides a median Lethal Dose (LD_{50}), *i.e*, the dose that causes death to 50% of the exposed organisms from a single dose of the test compound, along with any sublethal effects that may have occurred as a result of chemical exposure. An acute oral toxicity test is also required in Canada when potential exposure exists. The acute LD_{50} is assessed after 24 and 48 hours, but depending upon the outcome of the test, its duration can be extended up to a maximum of 96 hrs, if necessary. Based upon the outcome of the acute LD_{50} toxicity test, pesticides are classified as practically non-toxic, moderately toxic, or highly toxic to bees on an acute exposure basis. If the LD_{50} is less than 11 μg/bee, additional testing may be required in the form of a foliar residue study to determine the duration over which field-weathered foliar residues remain toxic to honey bees. On a case-by-case basis, additional higher-tiered studies such as field pollinator studies with honey bees (*i.e*., hive studies) may be necessary if the data from toxicity studies indicate potential chronic effects or adverse effects on colonies.

In the European Union (EU), risk to honey bees from exposure to pesticides is based on the European and Mediterranean Plant Protection Organization (EPPO) and includes a three-tiered progression of testing[4]. Guidelines describe laboratory tests, semi-field (cage/tunnel) tests, and field tests for evaluating the lethal and sub-lethal effects of pesticides on adult honey bees. The testing approach in the EU is similar to that of the U.S. and Canada in that it consists of a tiered approach, where laboratory studies are considered tier 1 tests, and semi-field and field tests are considered higher tiers. In contrast to the U.S., the EU and Canada requires the acute oral toxicity (LD_{50}) on adult workers in addition to the acute contact toxicity. In the EU, it is also standard practice to conduct both acute oral and acute contact LD_{50} studies on formulated end-use products, (in cases where exposure to the end use product itself is possible), as well as the technical grade (relatively pure) active substance.

[3] USEPA testing: OPPTS Guideline 850.3020; OPPTS 1996a

[4] Risk assessment: PP 3/10 (2) (OEPP/EPPO), test methodologies: guideline No. 170 (OEPP/EPPO); OECD 75

In addition to guideline toxicity test requirements, regulatory authorities around the world also make use of published open literature and dedicated studies of non-target arthropods to evaluate the potential effects of pesticides on terrestrial invertebrates, or as a line of evidence to require higher tiered testing. Along with guideline and open literature studies, adverse effect (*e.g.* bee kill incident) reports, and monitoring studies are considered in order to gauge the effects of pesticides on non-target organisms.

Risk Assessment for Systemic Compounds

Many who are familiar with pesticide risk assessment recognize that the methodology and testing scheme employed for foliar application products (where exposure may be primarily through surface contact) is not adapted well to assess potential risk from systemic pesticides. It was believed so because bees were subject to direct (pesticide) contact exposure during the use of many types of systemic treatments, such as those applied to the soil or as seed coats. However, with better understanding of the ability of these chemicals to be present in pollen and nectar during flowering, practicioners, researchers and regulators realized that systemic compounds present potential for both oral and contact exposure and, therefore, needed to be considered.

The EPPO has recently put forward a risk assessment scheme for systemic compounds that includes the same tiered testing system, but replaces the hazard quotient (HQ) calculation with a Toxicity Exposure Ratio (TER), where TER = PNEC/PEC. The PNEC is the Predicted No Effect Concentration, while the PEC is the Predicted Exposure Concentration. The PEC is determined from estimated or measured residue concentrations in the whole plant, flowers, pollen and/or nectar. The dose that individual bees might ingest is then calculated for different categories of honey bees (*e.g.*, larvae, queen, foragers) depending on the amount of contaminated pollen and nectar they may consume. PNECs are derived from acute, sublethal, and chronic toxicity data and may also include a factor to account for uncertainty. These factors range from 10 to 1 depending on whether the toxicity endpoint is assessed in a laboratory (Tier 1) or in a semi-field or field test, *i.e.*, uncertainty decreases as toxicity data become more representative of how the pesticide will be used.

Trigger Criterion and Levels of Concern

A "trigger criterion" is a value, a threshold, used to define the limit of risk that is consistent with protection goals. A trigger criterion or level of concern is compared to a quantitative risk estimate (*e.g.*, hazard quotient (HQ) employed in Europe, or a risk quotient (RQ) employed in North America) to determine if the estimated risk is acceptable or not. If the comparison between a level of concern and an estimated risk indicates that the use of a compound is inconsistent with protection goals, then it may be appropriate to either further refine the risk with additional data, or seek action to mitigate potential risk. (In Europe for example, when assessing a spray formula, the trigger criterion at the screening level is where HQ $\geq$50; such that when HQ $\geq$50, either higher tier data, or risk mitigation may be sought, in the US, estimates of risk (i.e., the risk quotient or RQ) is compared against the level of concern (i.e., LOC) to determine whether further refinement is needed.) Participants of the Workshop noted that while levels of concern promote efficiency in decision-making, risk assessment is an iterative process between risk assessors and risk managers, and is comprised of multiple lines of evidence in order to determine whether the use of a compound on a specific crop is consistent with a protection goal(s). Ultimately, trigger criterion and levels of concern are policy tools; and, as such, they are outside the realm of the SETAC Pellston Workshop and remain the right and responsibility of respective regulatory authorities to define.

CHAPTER 6 PROBLEM FORMULATION FOR AN ASSESSMENT OF RISK TO HONEY BEES FROM APPLICATIONS OF PLANT PROTECTION PRODUCTS TO AGRICULTURAL CROPS

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[1]Bayer CropScience LP, US; [2]Ministry for Food, Agriculture, and Fisheries, France; [3]Syngenta Ltd., UK; [4]Pest Management Regulatory Agency, Canada; [5]Environmental Protection Agency, US; [6]Department of Agriculture, US; [7]BASF Corporation, US

As mentioned previously, Phase 1 of the risk assessment process is problem formulation, (PF), where measurement endpoints are selected; a conceptual model is prepared that describes a risk hypothesis; and an analysis plan that articulated what data is needed and how it will be used to test the stated hypothesis is described. The problem formulation is intended to provide a foundation for the risk assessment, it articulates the purpose of the assessment, defines the nature of the problem (*i.e.*, potential for adverse effects given the nature of the chemical stressor and its existing and/or proposed use), and establishes the plan for analyzing available data and characterizing risk. Participants of the Workshop discussed the generic priciples of problem formulation and developed PFs for the assessment of risk of honey bees for two types of pesticide use scenarios: (1) application of a systemic chemical to the soil or seeds planted into the soil, and (2) application of a non-systemic chemical as a foliar spray. It should be noted there are other possible scenarios such as foliar spray application of a systemic chemical which may require a separate PF because both contact and oral exposure routes may be important. Likewise, some modification of the PF examples presented herein by the Workshop will likely be needed to apply them to non-Apis species in order to account for differences in behavior and life history. The goal here is to illustrate the process for developing a PF for assessment of pesticide risk to honey bees and other insect pollinators by providing some relevant examples.

What is a Problem Formulation?

Problem formulation is the first step of an ecological risk assessment (**Figure 1**). The objective of problem formulation is to develop a working risk hypothesis regarding the potential exposure to and resulting effects of a stressor (*e.g.*, a pesticide) on ecological receptors of concern (*e.g.*, honey bees). During problem formulation, objectives of the anticipated risk assessment are identified and underlying uncertainties and assumptions (constraints) regarding data are articulated. During problem formulation, initial scoping and integration of available information begins, and data/information gaps are identified. Within the context of a pesticide active ingredient being identified as a stressor, the problem formulation considers use information (*i.e.*, label information, formulations, application parameters (rates, methods, timing, *etc.*), crop types, information on target pests, *etc.*). (See Text Box below)

PF Questions: Assessing Available Information

<u>Source and Stressor Characteristics</u>

- What is the source of the stressor (anthropogenic, natural, point source, etc.)
- What type of stressor is it (chemical, physical, or biological)
- What is the intensity of the stressor (the dose or concentration, the magnitude or extent of the disruptions)
- What is the mode of action? How does the stressor act on organisms or ecosystem functions?

PF Questions: Assessing Available Information (continued)

Exposure Characteristics

- With what frequency does the stressor event occur (is it isolated, episodic, continuous)
- What is the duration of the exposure? How long does it persist in the environment? (half-life, does it bioaccumulate, dose it alter habitat, does it reproduce, or proliferate)
- What is the timing of exposure? When does it occur in relation to critical organism life cycle(s) or ecosystem events
- What is the spatial scale of exposure? Is the extent or influence of the stressor local, regional, global, habitat-specific or ecosystem-wide?
- What is the distribution? How does the stressor move through the environment?

Ecosystems Potentially at Risk

- What habitat types are present?
- How do these characteristics influence the susceptibility (sensitivity and likelihood of exposure) of the ecosystem to the stressor(s)?
- Are there unique features that are particularly valued (i.e., the last representative of an ecosystem type)
- What is the landscape context within which the ecosystem occurs?
- What are the geographic boundaries of the endpoint? How do they relate to the functional characteristics of the ecosystem/endpoint?
- What are the key abiotic factor(s) influencing the endpoint (e.g., climatic, geology, hydrology, etc.)
- Where and how are functional characteristics driving the ecosystem?
- What are the structural characteristics of the ecosystem (e.g., species number and abundance, trophic relationships)

Ecolgical Effects

- What are the type and extent of available ecological effects information (e.g., field surveys, laboratory tests, or structure-activity relationships)
- Given the nature of the stressor (if known), which effects are expected to be elicited by the stressor?
- Under what circumstances will effects occur?

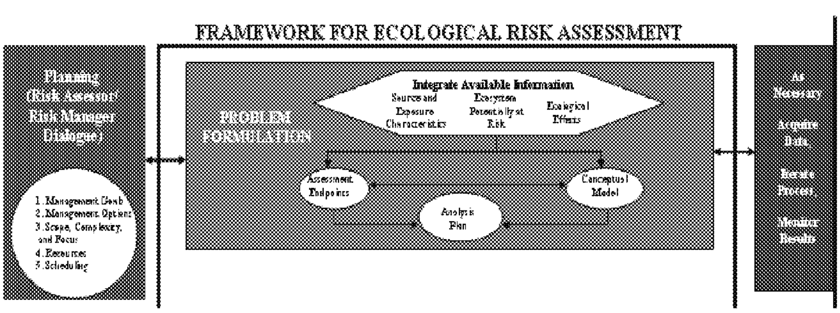

Figure [SEQ Figure * ARABIC]. Scheme depicting problem formulation phase of the ecological risk assessment process.

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Problem formulation has three deliverables (see middle box of **Figure 1**):

(1) risk assessment endpoints that adequately reflect management/ protection goals, and the ecosystem they represent;
(2) conceptual models that describe key relationships between a stressor and assessment endpoint; and,
(3) an analysis plan.

A critical component of problem formulation is planning dialog (left box of **Figure 1**) where risk assessors meet with risk managers and agree on management objectives and identify issues associated with the chemical. Problem formulation is intended to be iterative, and is informed by existing data (including open literature, existing data, or incident information). As more data become available, the risk hypothesis may change to reflect a more refined understanding of potential risks. The problem formulation identifies available data and information gaps and enables risk managers to convey potential limitations to registrants (chemical manufacturers who support labels) who may be able to provide information to address uncertainties.

Components of problem formulations include:

1) A description of the nature of the chemical stressor (typically a single technical grade active ingredient, but may include formulations, inerts or degradates of the active ingredient based on the availability of data);
2) A broad overview of pesticide existing/proposed uses;
3) A description of assessment endpoints, *i.e*., valued entitities (biological receptors) and their attributes, *i.e*. characteristics to be protected (survival, growth and reproduction), which are relevant to management/ protection goals;
4) A conceptual model which identifies the relationship between ecological entities and the chemical stressor under consideration. The conceptual model has two components, *i.e*., the risk hypothesis and conceptual diagram.
    a. The risk hypothesis describes the predicted relationships among the chemical stressor, exposure and assessment endpoint responses along with a rationale to support the hypothesis.
    b. The conceptual model diagram illustrates the relationships presented in the risk hypothesis and is typically represented by a flow diagram depicting the source (use), stressor, receptor and change in [endpoint] attribute.

5) An analysis plan is then presented to identify how the risk hypothesis will be assessed; it identifies data needs and methods for conducting the assessment and what measurements, *e.g.*, model-estimated environmental concentrations, no-observed adverse effect concentrations (NOAEC) and attribute changes, *e.g.*, foraging behavior, will be used

Selecting Assessment Endpoints

Assessment endpoints are explicit expressions of the actual environmental value that is to be protected. Selection of assessment endpoints begins to structure the assessment toward addressing management concerns. Assessment endpoints must be measurable ecosystem characteristics that represent management goals. Selection of ecological characteristics to protect becomes then, the basis for defining assessment endpoints, which connects broad protection goals with specific measures in risk assessment.

The element or characteristic of an ecosystem to be valued or protected must:

(1) have ecological relevance;
(2) be susceptible to known or potential stressors; and,
(3) be relevant to management goals and societal values.

Ecological Relevance

Ecologically-relevant endpoints reflect important characteristics of the system, and may be defined at any level of organization (*e.g.*, individual, community, ecosystem, landscape). Ecologically relevant endpoints often help sustain the natural structure, function, and biodiversity of a system or its components.

Ecologically-valuable endpoints are those that, when changed, cause multiple or widespread effects, (*i.e.*, are upstream of other effects in the ecosystem).

Susceptibility to Known or Potential Stressors

An ecological resource is susceptible when it is sensitive to a stressor, *i.e.*, it is affected by the stressor such as through a mode of action.

Sensitivity of an ecological resource may be relative to timing, *i.e.*, a life stage of an organism (or system). Sensitivity of an ecological resource may be affected by the presence of other stressors or natural disturbances.

Measures of sensitivity may include mortality, behavioral abnormalities, loss of offspring, habitat alteration, community structural change, and/or other factors.

Susceptibility (of an ecological resource) requires exposure [to a chemical stressor] such as through co-occurrence, contact, *etc*. Typically, the amount and conditions of exposure directly influence how an ecological resource will respond to a stressor.
Thus, timing of exposure, timing of effects, presence or absence of other stressors, and other variables add complexity to evaluations of sensitivity and/or susceptibility.

Relevance to Management Goals

Endpoints must be (i) scientifically valid, (ii) important to the public, and (iii) valued by risk managers (*i.e.*, reflect statutory obligations) in order for them to be relevant.

Risk assessors and risk managers should share their professional judgment when selecting and defining potential endpoints.

Defining Assessment Endpoints

Once ecological values are selected as potential endpoints (attribute changes), they must then be operationally defined. Two elements are required for operational definition:

(1) identification of the specific valued ecological entity, such as a species, or a functional group of species, or a community or ecosystem or specific habitat or unique place; and
(2) the characteristics (attributes) about the entity that is important to protect.

Assessment endpoints differ from management goals. Assessment endpoints should remain neutral and specific, whereas management goals represent a desired achievement (*i.e.*, a goal). As such, assessment endpoints do not contain words like "protect," "maintain," or "restore," or indicate a direction for change such as "loss," or "increase." Instead, assessment endpoints are ecological values defined for specific entities and their measurable attribute, providing a framework for measuring stress-response relationships.

Management goals and assessment endpoints are necessarily related. However, management goals must be appropriately scaled in order to be meaningfully represented by assessment endpoints.

For practical reason, it may be helpful to use assessment endpoints that have well-developed test methods, field measurement techniques, and predictive models. However, this is not necessary, since appropriate measures are identified during the development of the conceptual model and further specified in the analysis plan.

In situations where multiple stressors act on the structure and function of a [aquatic or terrestrial] community, an array of assessment endpoints that represent the community and ecological processes is typically more effective than a single endpoint.

Final assessment endpoint selection is an important risk manager-assessor checkpoint during problem formulation. Risk assessors and risk managers should agree that selected assessment endpoints effectively represent the management/ protection goals.

Common problems in selecting assessment endpoints are:

- the endpoint is a goal
- the endpoint is vague
- the ecological entity is better suited as a measure rather than an endpoint
- the ecological entity may not be sensitive to the stressor
- the ecological entity is irrelevant to the assessment
- the attribute is not sufficiently sensitive for detecting important effects (*e.g.*, survival compared with recruitment for endangered species).

Conceptual Models

Conceptual model(s) provide a written and visual representation of predictive relationships between ecological entities and the stressor(s) and may describe primary, secondary or tertiary exposure pathways, co-occurrences, ecological effects, or ecological receptors that are reflective of valued attribute changes in these receptors.

Multiple conceptual models may be developed to address several issues in a given risk assessment. When conceptual models are used to describe pathways of individual stressors and assessment endpoints and the interaction of multiple and diverse stressors and endpoints, more complex models and sub-models will often be needed.

Conceptual models are flexible and can be modified to accommodate new or additional data. For example, conceptual models can start out as broad and identify as many potential relationships as possible, then narrow as information is acquired. The complexity of a risk hypothesis is commensurate with the complexity of the risk assessment.

Conceptual models consist of two principal components:

(1) a set of risk hypotheses that describe predicted relationships among stressor, exposure, and endpoint response; and,

(2) a diagram that illustrates the relationship(s) presented in the risk hypotheses.

Diagrams are typically flow diagrams with boxes and arrows. Elements considered for inclusion in the diagram include: the number of relationships depicted; the comprehensiveness of the information; data abundance or scarcity; or the relative certainty of the pathway(s). Several smaller diagrams may be more effective than a single diagram that contains too much detail.

Diagrams should reflect/document a risk assessor's level of knowledge and degree of certainty regarding its components; and, should be discussed with risk managers to ensure that they reflect and communicate the managers concerns prior to analysis.

PF for a Systemic Chemical Applied to the Soil or as a Seed-dressing

Case 1: Problem Formulation for a Systemic Pesticide

Stressor description

Participants of the Workshop developed a risk assessment process through two case examples that were representative of two general types of pesticide delivery modes, systemic and foliar. Briefly outlined below is an example of a Problem Formulation for the pesticide risk assessment for pollinators first for a systemic compound, and then for a foliarly applied compound.

Stressor of concern is a systemic plant protection product (insecticide or acaricide) applied to the soil of field and orchard crops such as cotton, maize, oil-seed rape, wheat, barley, potatoes, sugar beets, cucurbits (*e.g.*, melons), citrus and pome fruit, or as a coating on seeds of field crops (cotton, maize, oil-seed rape, wheat, barley). Crop plants absorb the chemical through the roots and translocate it into above ground tissues of the plant. Plant magnitude of residue studies demonstrate that both the parent compound and a primary degradate with insecticidal properties comprise the residues found in treated plants. Use of the product provides effective control of several economically important chewing and sucking pest insects such as aphids, psyllids and white flies. Application timing is at planting or during transplant of field crops and after flowering of orchard crops.

The above paragraph covers the first two components of a PF, which were listed above as (1) a description of the nature of the chemical stressor, and (2) a broad overview of pesticide existing/proposed uses. The third component of a PF is a description of assessment endpoints, *i.e.*, valued entities (biological receptors) and their attributes, *i.e.* characteristics to be protected (*e.g.*, survival, growth and reproduction), which are relevant to protection goals.

Management goals

As discussed above, protection goals are policy decisions that are set by government agencies and other organizations that represent the interests of the societies they serve. In the absence of specific protection goals, the participants used those developed during the workshop, these included;

- Protection of pollination services provided by *Apis* and non-*Apis* species'
- Protection of honey production and other hive products; and,
- Protection of pollinator biodiversity,

The first of these statements is applicable to pollinators (*Apis* and non-*Apis*) in general. The second and third statements are applicable to non-managed pollinators.

Assessment endpoints

For honey bees, logical assessment endpoints are colony strength (population size and demographics) and colony survival (persistence). Since a colony loss simply represents the situation when colony strength is minimal, it could be argued that *colony survival* is not needed as a separate assessment endpoint. Various measures of colony strength are often made when bee hives are rented and placed at agricultural crops. Rental fees are greater for strong colonies than weak colonies because colony strength is expected to be related to the quality of pollination service provided by the colony. Colony strength will likely be significantly impacted if queen viability, brood development or general worker bee health is negatively impacted for an extended period of time. There are many known cases where pesticide exposure has caused effects on colony strength. Colony strength appears to meet very well the previously listed criteria for an assessment endpoint. Colony strength:

(1) has ecological relevance,
(2) is susceptible to known or potential stressors, and,
(3) is relevant to the management/ protection goals and societal values associated with maintenance of pollination services.

Conceptual Model

The fourth component of PF listed previous is the conceptual model which identifies the relationship between ecological entities and the chemical stressor under consideration. The conceptual model has two components, *i.e.*, the risk hypothesis and conceptual diagram.

Risk Hypothesis

The risk hypothesis describes the predicted relationships among the chemical stressor, routes of exposure and effects along with a rationale to support the hypothesis.

For a systemic pesticide applied to the soil or as a seed dressing, the risk hypothesis involves the following logical steps describing how exposure most likely occurs and results in effects on the assessment endpoint (colony strength). The hypothesis is:

1) the use of the systemic plant protection product results in toxic concentrations in nectar, pollen or other parts of plants visited by honey bees,
2) forager honey bees collect the contaminated nectar and pollen and transport it back to the hive where it is incorporated into the food stores of the colony,
3) Forager, hive bees, bee brood and the queen are exposed to concentrations of the chemical mainly via oral ingestion,
4) If the exposure concentration is high enough, toxic effects on forager bees, hive bees, bee brood and/or the queen result in reduced queen fecundity, brood development success or survival of adult bees.
5) Colony strength is affected as a result of reduced fecundity, brood success or adult survival.

The duration of exposure of forager bees will depend on the persistence of the chemical in the soil and inside of treated plants, the duration of bloom, and the chronology of application (planting of treated seeds or application to the soil) of the chemical to agricultural fields within the landscape around the hive. If the hives are moved from site to site to provide pollination services, as is common in the U.S. for honey bees, there may be repeated opportunities for exposure. For hive bees, exposure may occur over a relatively long period of time since residues are incorporated in the hive's food stores. The persistence and concentration of the chemical in stored food (e.g., honey and bee bread) will influence the exposure profile. Chemicals that rapidly degrade under these conditions will have less potential to cause chronic toxicity.

Based on the risk hypothesis, key questions that need to be answered during risk analysis are:

1) To what extent do foraging honey bees visit treated plants and collect materials (pollen, nectar, *etc*.) that may contain residues of the chemical being assessed?
2) What levels of the parent compound and the toxic metabolite are present in materials (pollen, nectar, *etc*.) collected by honey bees?
3) How do the above concentrations change over time, especially in collected pollen and nectar and in hive-stored pollen and nectar?

4) What concentrations in pollen and nectar when fed to a bee colony result in a significant decrease in queen fecundity, brood success, adult survival, and ultimately, colony strength?

Conceptual Model Diagram

The conceptual model diagram depicted in **Figure 2** below illustrates the relationships presented in the risk hypothesis for the assessment of risk of a systemic pesticide applied to the soil or as a seed dressing.

The source of exposure is application of the systemic plant protection product to the soil or as a coating to seeds planted in the soil. The primary routes of exposure are assumed to be via residues in pollen and nectar (yellow boxes); however other routes of exposure such as ingestion of residues in surface water, plant exudates (*e.g.*, guttation fluid), and abraded seed dust are included also. Primary routes of residue transfer are indicated by thick arrows, lesser routes by thin arrows. Forager worker bees may be exposed by both contact and oral ingestion, however since the chemical is applied to the soil, potential for contact exposure is assumed to be limited. The main route of exposure for worker bees is hypothesized to be the oral route, particularly the ingestion of nectar, since nectar is the primary food consumed by forager worker bees. Pollen is also collected on hairs on the forager worker bees' bodies, or in small pouches (pollen baskets) on their hind legs. The nectar and pollen collected by worker bees are brought back to the hive where they are incorporated into the food stores consumed by hive bees which in turn use them to produce food for the queen and developing brood. If the pesticide concentration is high enough, toxic effects on forager bees, hive bees, bee brood and/or the queen may result in reduced queen fecundity, brood development success or survival of adult bees. If these effects are severe enough and/or last long enough, a significant effect on colony strength may result.

[SHAPE * MERGEFORMAT]

Figure 2. Depiction of stressor source, potential routes of exposure, receptors and attribute changes for a systemic pesticide applied to the soil or as a seed dressing.

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Analysis Plan

The final component of the PF is the analysis plan. The analysis plan identifies how the risk hypothesis will be assessed. It identifies data needs and methods for conducting the assessment and what measures of exposure (*e.g.*, estimated environmental concentrations, monitoring data) and measures of effects (*e.g.*, no-observed adverse effect concentrations (NOAEC) and attribute changes (*e.g.,* individual bee mortality, colony strength attributes might include estimates of the percent coverage of hive frames by adult bees, open brood and capped brood) will be used. The intent here is to provide only a one possible example of an analysis plan.

Data Needs for Exposure Characterization

While it may be possible to develop a computer model to predict residues of systemic chemicals in various plant tissues, such models are not currently available; and, direct measurements are obtained through field studies. For the purposes of this problem formulation, let us assume that field studies have been conducted to measure residue levels of the parent compound and the toxic degradate in pollen and nectar collected from treated plants by honey bees. These measurements can be used to determine the median (50%tile) and high end (defined here as the 95%tile) concentrations expected to be present in pollen and nectar. Estimated daily intake rates for pollen and nectar by various castes of honey bees listed in **Table 1** of Rortais *et al*. (2005) may be used to convert food concentrations (µg chemical/g of food) to a daily dose (µg chemical/individual bee/d). Some toxicity endpoints are expressed in units of a test concentration (*e.g.*, µg chemical/kg test matrix = parts per billion or ppb); others as a dose (*e.g.*, µg chemical/individual bee). The units of the measure of exposure must match the units of the measure of toxicity in order for a valid risk estimate to be calculated.

Data Needs for Effects Characterization

As described briefly in Chapter X. the progression of effects data development is to begin with standard laboratory assays and, as necessary, conduct higher tier studies which may consist of specialized laboratory, semi-field and/or field tests. In this sort of testing sequence, the results of higher tier studies [provided they are of sufficient quality and reliability] are used to refine the overall conclusions about risk.

Because the main route of exposure expected for systemic chemicals is oral ingestion, toxicity testing of the oral route of exposure is needed to characterize potential effects of residues in bee foods. Standard protocols are available for conducting acute but not chronic oral toxicity tests. Food with residues of systemic compounds may be stored in the hive and used by the colony for long periods of time. The development of a standardized chronic feeding test may be needed. A 10-day feeding test of individual adult honey bees has been proposed by the International Commission on Plant-Bee Relationships (Alix *et al*., 2009) as a means to define a chronic toxicity measure. Alternatively, experiments in which whole colonies are fed prescribed concentrations of the test chemical for periods ranging from weeks to months have been performed with some systemic chemicals. Measures of effects of these various chronic tests have included the median lethal concentration and the NOAEC for various colony attributes, including colony strength (percent frame coverage with adult bees, open brood, capped brood, etc.).

If unacceptable risks cannot be discounted on the basis of simple laboratory test results, and conservative exposure assumptions, then higher tier studies may be conducted to determine the likelihood and severity of risks under conditions simulating actual agricultural use. Semi-field (tunnel) and field studies may have the advantage of evaluating all routes of exposure simultaneously under conditions reasonably similar to actual field use, whereas laboratory studies are generally limited to evaluation of a single route of exposure under artificial conditions.

Risk Characterization Approach

Most assessments of ecological risks of pesticides use a conventional risk quotient (RQ) or toxicity-exposure ratio (TER) approach that compares point estimates of exposure (*e.g.*, typical and high end estimates of residue levels in various food types) to estimated thresholds of toxicity (*i.e.*, median lethal concentration or NOAEC). The RQ equals the exposure point estimate divided by the toxicity point estimate. Although RQ values are dimensionless numbers, the greater the RQ, the greater is the presumed risk. TERs are the reciprocal of the RQ, so the greater the TER, the lower the risk. Regulatory agencies compare the RQ or TER to an established level of concern (LOC) that is presumed to represent a threshold between minimal and non-minimal risk. If the RQ is less than the LOC, or the TER is greater than the LOC, the risk may be presumed to be minimal and further testing is unnecessary provided the constituent elements of the RQ are considered to be sufficiently inclusive. Risk assessment is iterative with screening-level point estimates of exposure and toxicity often used in initial assessments. If the RQ of a screening-level assessment exceeds the LOC, the conclusion is the risk is potentially non-minimal, and further testing may be appropriate to clarify the risk. If semi-field and/or field tests are performed, these results may be incorporated into the risk characterization (provided the studies are of sufficient quality) using a weight-of-evidence approach.

Case 2: Problem Formulation for a Contact (aka Knock-down) Pesticide

Stressor description

The stressor of concern is a "knock-down" insecticide product applied as a spray to field and orchard crops such as cotton, maize, vegetables, citrus and pome fruit to control pest insects that feed on stems, leaves, inflorescences and fruit. Being a non-systemic chemical, it does not penetrate treated plant surfaces and so it is not translocated systemically throughout the plant. For the purposes of this example, let's assume residues on plant foliage dissipate fairly rapidly, with a foliar dissipation half-life of 2-3 days. Because of the short residual toxicity, several applications may be necessary to protect plants during critical phases of the growing season. Based on their chemical structure, none of the chemical's major break down products are expected to exhibit significant toxicity to insects. The product label recommends application rates that vary from 20 to 30 g active ingredient (a.i.) per hectare (ha), depending on crop and growth stage. It cautions against making applications to flowering crops when foraging honey bees are likely to be present. If applications are needed during bloom, it is recommended they occur in the late afternoon or evening when bee foraging activity is relatively low.

Management Goals

As discussed above, protection goals are policy decisions that are set by government agencies and other organizations that represent the interests of the societies they serve. In the absence of specific protection goals, the participants used those developed during the workshop, these included;

- Protection of pollination services provided by *Apis* and non-*Apis* species'
- Protection of honey production and other hive products; and,
- Protection of pollinator biodiversity,

The first of these goals is applicable to pollinators in general. The second, and third statement is applicable to an assessment focused on managed honey bees.

Assessment Endpoints

For honey bees, logical assessment endpoints include colony strength (population size and demographics) and colony survival (persistence). Since a colony loss simply represents the situation when colony strength is minimal, it could be argued that *colony survival* is not needed as a separate assessment endpoint. Various measures of colony strength are often made when bee hives are rented and placed at agricultural crops. Rental fees are greater for strong colonies than weak colonies because colony strength is expected to be related to the quality of pollination service provided by the colony. Colony strength will likely be significantly impacted if queen viability, brood development or general worker bee health is negatively impacted for an extended period of time. There are many known cases where pesticide exposure has caused effects on colony strength. Colony strength appears to meet very well the previously listed criteria for an assessment endpoint. Colony strength

(1) has ecological relevance;
(2) is susceptible to known or potential stressors; and,
(3) is relevant to management/ protection goals and societal values.

Conceptual Model

The fourth component of PF listed previously is the conceptual model which identifies the relationship between ecological entities and the chemical stressor under consideration. The conceptual model has two components, *i.e.*, the risk hypothesis and conceptual diagram.

Risk Hypothesis

The risk hypothesis describes the predicted relationships among the chemical stressor, exposure and assessment endpoint responses along with a rationale to support the hypothesis.

For a non-systemic pesticide applied as a foliar spray, the risk hypothesis involves the following logical steps describing how exposure most likely occurs and results in effects on the assessment endpoint (colony strength). The hypothesis is:

1) residues in spray droplets may (1) contact bees directly (i.e., bees hit directly by the spray) or (2) be deposited in water (*e.g.* puddles) from which bees drink, or (3) be deposited on plant surfaces visited by honey bees,
2) spray deposits hitting open flowers may contaminate nectar and pollen sources for a short period of time post-application (until these flowers are replaced by others that were not open during spray),

3) forager honey bees may ingest contaminated water and/or contaminate nectar, and may collect and transport back to the hive contaminated nectar and pollen where these latter materials are then incorporated into the food stores of the colony,
4) If the exposure concentration is high enough, toxic effects on forager bees, hive bees, bee brood and/or the queen may result in reduced survival of adult bees, brood development or queen fecundity,
5) Colony strength is affected as a result of reduced fecundity, brood development or adult survival if these effects are severe enough or last long enough,
6) Since the chemical is knock-down insecticide with short residual time on foliage, the primary effect expected may be direct mortality of forager worker bees shortly after spraying (*i.e.*, a bee kill event).

The duration of exposure of forager bees will depend on the persistence of the chemical on plant surfaces, and the persistence (duration of bloom) of individual flowers that were hit by the application. As new blooms replace old ones, the potential for exposure may rapidly decrease. Thus, the main concern for foliar spray applications has traditionally been acute exposure of forager worker bees that results in a discreet bee kill event. However the possibility of residues in bee-collected pollen and nectar being brought to and stored in the hive should be considered since this scenario may lead to chronic exposure of the hive bees, queen and bee brood.

Based on the risk hypothesis, key questions that need to be answered during risk analysis are:

1) To what extent are forager honey bees active when spray applications are made?
2) If forager bees incur contact exposure during or shortly after application, are the levels of exposure great enough to cause “knock-down” intoxication?
3) If spray deposits represent an initial lethal hazard to honey bees, how long does this situation last?
4) To what extent do foraging honey bees visit sprayed plants and water sources and collect materials (pollen, nectar, *etc.*) that may contain residues of the chemical?
5) What levels of the chemical are present in materials (pollen, nectar, etc.) collected by honey bees and brought back to the hive?
6) How do the above concentrations change over time, including changes in concentrations in hive-stored pollen and nectar?

7) What concentrations in pollen and nectar when fed to a bee colony result in a significant decrease in queen fecundity, brood development, adult survival, and ultimately, colony strength?

Conceptual Model Diagram

The conceptual model diagram depicted in **Figure 3** below illustrates the relationships presented in the risk hypothesis for the assessment of risk of a non-systemic chemical applied as a foliar spray.

The source of exposure is foliar spray application of the non-systemic plant protection product to crop plants. The primary routes of exposure are assumed to be via contact of foraging worker bees with spray as it is applied or with freshly-deposited residues on plant surfaces. For flowers open during spraying, residues may occur in pollen and nectar, and these materials may be brought back into the hive and stored as food that is later utilized by hive bees, bee brood and the queen. Another possible route of exposure is via surface water (e.g., puddles) that are oversprayed and used by bees as a source of drinking water. Primary routes of residue transfer are indicated by thick arrows, lesser routes by thin arrows. Greatest exposure is expected for forager worker bees which may be exposed via contact with spray droplets and residues on plant surfaces, and via ingestion of residues in water and nectar. If the exposure level is great enough, enough forager bees may be killed that colony strength is reduced (e.g., large bee kill event).

Bees in the hive could also be exposed, but the exposure levels are not expected to be as great as for forager bees unless the hive is inadvertently sprayed (overspray) during application. However, if the residue loads on the bodies of forager bees (which may be ingested by hive bees during social grooming) and/or the concentration in pollen and nectar brought into the hive are high enough, toxic effects on hive bees, bee brood and/or the queen may result. If these effects are severe enough and/or last long enough, a significant effect on colony strength may result.

[SHAPE * MERGE Foliar Spray Application

Figure [SEQ Figure * ARABIC]. Depiction of stressor source, potential routes of exposure, receptors and attribute changes for a nonsystemic pesticide applied as a foliar spray.

Analysis Plan

The final component of the PF is the analysis plan. The analysis plan identifies how the risk hypothesis will be assessed. It identifies data needs and methods for conducting the assessment and what measures of exposure (*e.g.*, estimated environmental concentrations) and measures of effects (*e.g.*, no-observed adverse effect concentrations (NOAEC) and attribute changes (*e.g.,* colony strength attributes might include estimates of the percent coverage of hive frames by adult bees, open brood and capped brood) will be used. Different workgroups in the Pellston Workshop will review and issue detailed reports on the various measures of exposure and measures of effect that could be used, and make specific recommendations for future testing needs in order to obtain the necessary data. The intent here is to provide only a one possible example of an analysis plan.

Screening Assessment

A simple Hazard Quotient approach is currently used in Europe to predict whether foliar applications of plant protection products have the potential to cause observable bee kills. This screen is has been validated by comparing predictions to results of field studies and incident monitoring programs (see Mineau *et al.* 2008).

The HQ calculation is made as follows:

HQ = application rate (g a.i./ha) / LD_{50} (µg/bee)

If HQ < 50, a minimal risk may be presumed
If HQ > 50, a non-minimal risk cannot be excluded (more testing needed)

For example, let's assume an acute contact toxicity study has been conducted and the LD50 for the chemical in question is 0.1 µg/bee. Using the maximum application rate of 30 g ai/ha, the HQ calculation would be 30/0.1 = 300. Since this value is greater than 50, the risk of bee kills can not be discounted as minimal. Further assessment is needed to evaluate risk.

Data Needs for Refined Exposure Characterization

The label statement prohibiting application to crops during bloom until the evening or night time hours should go a long ways toward eliminating the possibility that foraging bees will be hit by the spray droplets as they are applied to the crop. A key piece of information needed is how long residues on sprayed vegetation remain toxic to visiting honey bees. This could be estimated from field studies that measure the magnitude and dissipation of residues on sprayed vegetation. It may be simpler to determine this using a standard EPA Tier 2 bioassay (discussed in greater detail below). Another key piece of information is to determine the residue levels in plant materials (mainly pollen and nectar) collected by forager bees and brought in to the hive. It may be necessary to conduct field studies to obtain direct measurements. Such measurements can be used to determine the median (50%tile) and high end (e.g., 95%tile) concentrations expected to be present in pollen and nectar. Estimated daily intake rates for pollen and nectar by various castes of honey bees listed in Table 1 of Rortais *et al.* (2005) may be used to convert food concentrations (ug chemical/g of food) to a daily dose (µg chemical/individual bee/d). Some toxicity endpoints are expressed in units of a test concentration (*e.g.*, µg chemical/kg test matrix = ppb); others as a dose (*e.g.*, µg chemical/individual bee). The units of the measure of exposure must match the units of the measure of toxicity in order to for a valid risk estimate to be calculated.

Data Needs for Effects Characterization

The logical progression of effects data development is to begin with standard laboratory assays and as necessary conduct higher tier studies which may consist of specialized laboratory, semi-field and/or field tests. In this sort of testing sequence, the results of higher tier studies are used to refine the assessment and are weighted more heavily in reaching overall conclusions about risk.

Because the main route of exposure for forager bees is expected to be contact, the standard EPA Tier 2 bioassay with honey bees seems appropriate. In this test, groups of honey bees are exposed via contact to vegetation which was sprayed in the field and then collected for testing after prescribed time intervals. For example, a common protocol is to evaluate the contact toxicity of vegetation at 2, 8 and 24 hours post-application. In the case of this chemical, let's assume it was found that a high level of mortality occurred in bees exposed to 2-h old foliar residues, but that normal honey bee survival was noted when bees were exposed to foliar residues collected 8 and 24 hours after application. This indicates there is window of acute hazard from acute contact that exists for 2-8 hours post application.

To assess the significance of residues in pollen and nectar that may be brought in to and stored in the hive, oral toxicity testing is needed. At a minimum, an acute oral toxicity test can be used to establish oral dose levels that are potentially lethal to adult bees. If there are indications that significant residues will be contained in hive stored food (pollen, honey), then a chronic feeding study may be needed to identify the no observed adverse effect concentration. A 10-day feeding test of individual adult honey bees has been proposed by the ICPBR as a means to define a chronic toxicity measure. Larval bees are more sensitive than adult bees to some classes of chemicals. Various kinds of larval feeding tests have been developed to establish dose levels that affect larval survival and development. Alternatively, experiments in which whole colonies are fed prescribed concentrations of the test chemical for periods ranging from weeks to months have been performed with some chemicals. Measures of effects directly related to colony strength can be obtained from such studies.

If adverse effects cannot be discounted on the basis of simple laboratory test results, higher tier studies may be conducted to determine the likelihood and severity of effects under conditions simulating actual agricultural use. Semi-field (tunnel) and field studies may have the advantage of evaluating all routes of exposure simultaneously under conditions reasonably similar to actual field use, whereas laboratory studies are generally limited to evaluation of a single route of exposure under artificial conditions.

Risk Characterization Approach

Calculation of the screening assessment HQ represents an initial risk characterization of the chemical. If the HQ < 50, there is a presumption of minimal acute risk in the EU, based on historical investigations of bee kill incidents (Mineau *et al*. 2008). Based upon the results of the acute toxicity test and the use pattern, tier 2 tests may be required by the EPA, which may provide some insight into whether the label statement requiring applications be made in late afternoon or evening will mitigate the potential risk. Since, in our example, this study showed residual toxicity last less than 8 hours, residues from applications made in the late afternoon or evening should not pose an acute hazard to bees that begin foraging the following day. A RQ or TER could be calculated to assess the risk posed by residues in pollen and nectar. The RQ or TER calculation would compare the concentration measured in these matrices or dose taken in by various castes of bees to available toxicity endpoints (LD_{50}, no-observed-adverse-effect concentration, etc.). Finally, well-designed semi-field or field studies may provide the most reliable information regarding the level of risk actually occurring under field use conditions. A weight-of-evidence approach may be taken to integrate the various lines of evidence.

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Chapter 7 Assessing Exposure of Pesticides to Bees

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Introduction

An essential component of an ecological risk assessment is a prediction of exposure of the organisms being assessed. In this chapter outlines exposure pathways, identified by the Participants, from both non-systemic and systemic pesticides, and discusses methods used to predict pesticide exposure to honey bees and non-*Apis* bees. This chapter also provides an outline of techniques employed to measure pesticide residues in relevant matrices, and discusses higher-tier field study designs that are used to refine bee exposure assessments for specific products. Finally, this chapter presents perspectives regarding pesticide application technologies that can be employed to mitigate bee exposure, as well as future research needs to further refine exposure assessments for this taxa.

Potential Routes of Exposure for Honey Bees to Pesticides

Managed honey bees provide pollination service for many insect-dependent pollinated crops (Morse and Calderone, 2000) as well as providing pollination to native and non target plant species (National Academy of Science, 2006). In addition, bee keepers utilize honey bees for honey production on cropping systems and natural environments within widely varied landscapes. Although honey bees are recognized as the most important commercial pollinator, native bee species also play an important role in pollination of crops and native plant species (Prescott-Allen and Prescott-Allen, 1986; Maeta, 1990; Kremen *et al*., 2004; Greenleaf and Kremen, 2006a; Losey and Vaughan, 2006; Winfree *et al*., 2008). Because of the variety of ways that pesticides are applied, there are several potential routes of exposure for foraging bees as well bees in hives and nests.

Foraging honey bees and foliar sprayed pesticides

Honey bees can be exposed to direct spray, or through contact with the crop that a pesticide is applied to. Bees can be exposed to pesticides that drift to plants on the edges of the treated field, potentially leading to either dermal or oral exposure, as well as water sources near the treated field which may contain residues either from drift or surface run-off. Pesticide drift can also reach hives directly if the hives are located in or near a treated field.

When foliar applications are made directly onto flowers, oral exposure can occur through the collection of contaminated pollen, nectar, or honeydew and/or by contact exposure if the product is directly sprayed on foraging bees or the plant parts that they can come in contact with during foraging.

Foraging honey bees and systemic pesticides

Honey bees can be exposed to systemic pesticides through the following routes:

- Via fugative dust released from treated seed (Alix *et al.*, 2009c). The exposure can be oral and/or contact from bees foraging on flowers upon which abraded dust falls; also, bee may be exposed if it flys through the dust or vapors, or if the bee is foraging on weeds and flowers (i.e., understory or in material that is adjacent to the target site) covered with contaminated dusts.

- Via contaminated pollen and nectar. Pollen and nectar of plants grown from treated seed or soil applications (including ground drench or chemigation applications) may contain levels of the pesticide. Potential residues of systemic pesticides in pollen and nectar might be collected by foragers and brought back to the hive to be stored, processed and fed to adults and larvae.

- Via residues in rotational crops or alternative forage (understory or adjacent areas) that may take up and express pesticide residues. Even if target crops are not attractive to bees, systemic compounds that are persistent in soil may represent potential exposure through residues in the nectar and pollen of the succeeding (rotational) crop or associated weeds. Potential residues of systemic pesticides in pollen and nectar may be collected from plants which have taken up the systemic pesticide. Presence of pesticide residues in a succeeding crop may be influenced by the type of crop, treatment pattern, the physicochemical properties, and environmental fate of the compound

- Pesticides can be distributed through microencapsulated technology. Micro-encapsulated formulations are designed to adhere, through the use of a sticking agent, on the foliar part of the plants or applied directly on the soil. Microencapsulation formulation technology is used to control exposure by slowly releasing the pesticide,,\ reducing drift, and reducing human exposure. Honey bees can potentially be exposed to certain micro-encapsulated pesticides if the micro-capsules are of similar size to pollen. Bees may inadvertently collect the micro-capsules and bring them back to the hive. If the microcapsules are collected by honey bees and mixed into the beebread, the exposure may affect the whole colony as the pesticide may be fed to the larvae. Such incidents have been reported following the use of Pencap-M, a micro-encapsulated formulation of methyl-parathion (Mason, 1986).

- Other potential routes of exposure for foraging bees include inhalation (Seiber and McChesney, 1987; Seiber *et al.*, 1991), and consumption of aphid honey dew, guttation water (Girolami *et al.*, 2009), or chemigation water from soil treatments.

Non-foraging adult honey bees (e.g., nurse bees, queens, drones)

All cast members of a colony may be potentially exposed to contaminants through the wax which composes their hive. Larvae are reared in cells made of beeswax, and as adults, they are in constant contact with the wax while they are in the hive. After pupation, bees chew through the wax coating on the brood capping and emerge as an adult. During colony development, worker bees continuously modify the wax cell structure (*e.g.*, converting male cells into worker cells, cleaning brood cells to stock honey and vice-versa). Pesticides that are lipophilic tend to accumulate in wax (Tremolada *et al.*, 2004). If the beeswax contains pesticide residues, honey bees, especially larvae, may be subject to contact exposure, depending upon the bioavailability of the pesticide (Chauzat *et al.*, 2007)

Nurse bees

For the first one to three weeks after emergence, adult worker bees remain in the hive to perform many duties including, but not limited to, feeding and cleaning larvae, cleaning cells, building new cells, processing and storing nectar, packing pollen, and capping cells. Nurse bees process pollen and nectar into beebread and honey, respectively, and also produce larval jelly. Nurse bees are the only cast/life-stage of honey bees that consume significant amounts of raw pollen, which is regurgitated and processed into beebread. (Beebread is then stored in the hive until it is processed by nurse bees into brood food and fed to larvae.) Bees can potentially be exposed to pesticides during all of these activities if residues are brought back to the hive by foraging bees.

Nurse bees may be potentially exposed to higher pesticide residues than larvae as they process pollen into larval food. In addition, nurse bees can potentially be exposed to pesticides through water brought back to the hive for cooling and brood rearing. Nurse bees may also be exposed as they process nectar into honey within beeswax cells as well as through contacting wax while moving through the hive. Pesticides applied directly to the hive for *Varroa* sp. control and other pests are a direct route of exposure to nurse bees (Martel *et al*., 2007).

Drones

Drone larvae receive more food than worker larvae, but its composition is similar (Free, 1977). Upon emergence as adults, drones receive food from worker bees or by feeding on stored honey. Similar to larvae and nurse bees, drones may be exposed to pesticides through food or residues within the hive.

Queens

Larvae that continue to only to be fed royal jelly beyond three days develop into queens (Free, 1977). A queen may live within the hive from 6 months to several years. Therefore, the queen may be exposed to multiple pesticides and residues within the hive over a relatively long period of time. Feeding on royal jelly and contact with residues in the hive are the major potential routes of contaminant exposure for queens.

Honey bee larvae

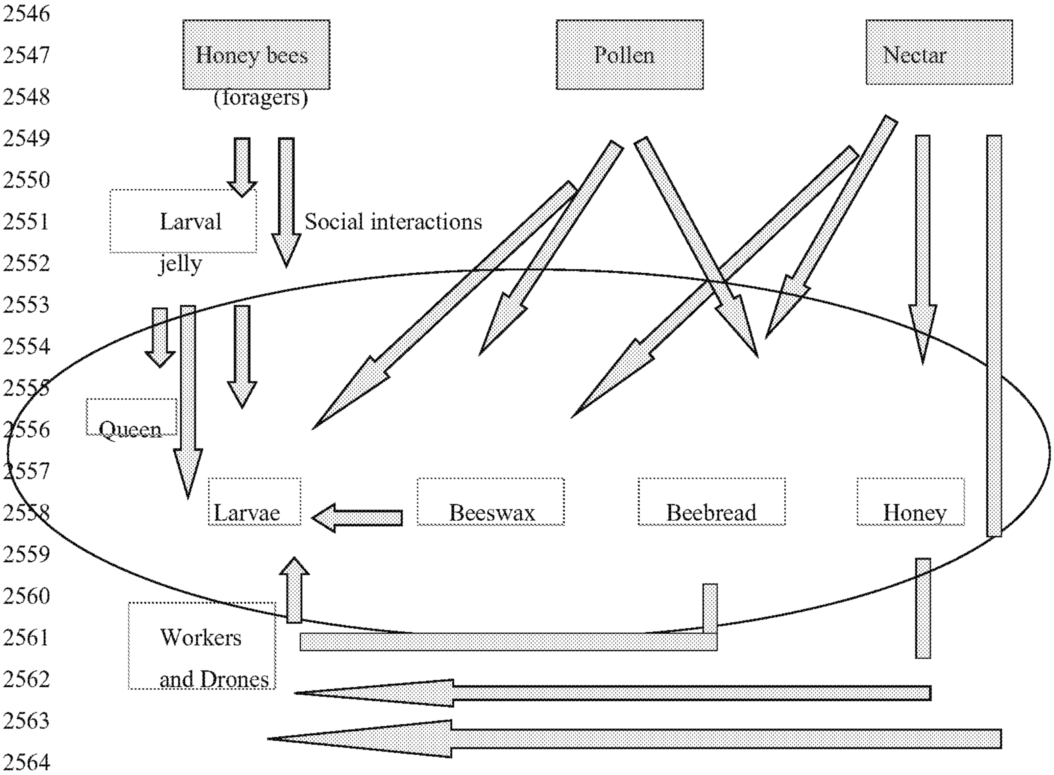
Honey bee larvae can be exposed to pesticides through ingestion of contaminated pollen, beebread, honey and larval jelly. Larval worker bees are fed larval jelly (also referred to as worker jelly or royal jelly) for three days after egg hatch. Larval jelly is a glandular secretion from the nurse bees' hypopharyngeal glands that consist of some white components (mostly lipids) and clear secretion (Free, 1977). Honey bees exposed to some pesticides can potentially produce contaminated larval jelly (Tremolada *et al*., 2004) that could be fed to the queen, workers and the larvae. From the fourth to the sixth day after egg hatch, worker larvae are fed bee bread, which is largely processed pollen, but also includes some larval jelly, honey, and pollen (Free, 1977). The beebread can be contaminated if processed with contaminated pollen (Orantes Bermejo *et al*. 2010).

Water is brought back to the hive and used to cool the hive, dilute stored honey, and prepare larval food. If pesticide residues are present in this water, larvae may be exposed through direct contact to the water or through ingestion of food prepared with the water. Larvae may also be exposed via contact exposure to pesticides that accumulate in wax or from residues on foraging bees. Additionally, larvae, as well as adults, may be exposed to insecticides/miticides applied directly to the hive by the beekeeper for *Varroa* sp. control and/or fungicides, bactericides or any other active substance applied for disease control.

Residue movement through the hive

Pesticides can be transferred to the hive environment from foraging honey bees which bring residues back to the hive in contaminated pollen and nectar. All potential transfer and movement of a pesticide in a hive is highly dependant on use pattern of the pesticide product, as well as the physical and chemical properties of the contaminants. Some chemicals may persist in the hive, resulting in prolonged exposures, while others dissipate and/or degrade into metabolites. Some pesticide metabolites can also be toxic to honey bees (Suchail *et al*., 1999; Martel *et al*., 2011). Therefore, while research continues to shed light on the fate and movement of a compound in a hive, it is important to understand and consider the fate properties of a compound in assessing potential exposure. Below is a conceptual model of exposure routs for pesticides to honey bee colonies.

Conceptual Model showing how contaminants may potentially reach various matrices within honey bee colonies



Honey bees (i.e., foragers carrying contaminated dust), pollen and nectar are the three main sources for in-hive contamination. Arrows show potential major contamination transfer routes. For minor routes, please refer to the text.

Potential Routes of Exposure for Non-*Apis* Bees

Most routes of exposure that have been examined for honey bees are valid for non-*Apis* bees as well, however, because of their diverse and often unique biology, non-*Apis* bees are also prone to other routes of pesticide exposure. Understanding different exposure routes is important because it is not feasible to conduct tests on the more than 20,000 species of non-*Apis* bees worldwide (Michener, 2007; [HYPERLINK "http://www.discoverlife.org/%20mp/20q?guide=Apoidea_species"]). Therefore, a risk assessment for non-*Apis* bees will be based mainly on the exposure routes reviewed here for honey bees, and tailored for different species groups. If more specific exposure information is required for risk assessment refinements, actual measures of unique exposure pathways may be adapted from tests conducted on some key, non-*Apis* species (see section below on Higher Tier Studies). Because of the large diversity of non-*Apis* biological features, this section will be structured around some broad features of non-*Apis* bee ecology.

Nesting sites and nesting materials

Social non-*Apis* bees, such as stingless bees (*e.g.*, *Melipona* spp. and *Trigona* spp.) nest in natural and human-made cavities that are usually located above ground. However, plant resins used by these bees for nest construction may be contaminated by pesticide applications (Romaniuk *et al*., 2003). Honey bees collect resins mainly from tree buds (*e.g.* cottonwoods (*Populus deltoides*)) to make propolis to waterproof their nests and seal up cracks and holes. Similar to honey bees, stingless bees, solitary orchid bees (Euglossini) and some leaf-cutter (megachilid) bees, collect resins to waterproof their nests and seal holes, but certain non-Apis species also use these resins as an important structural component of nest building (Murphy and Breed, 2008; Roubik, 1989). Tropical non-*Apis* bees usually collect their resin from flowers of the family Clusiaceae and Euphorbiaceae (Murphy and Breed, 2008).

Most bumblebee species, (a semi-social species) such as *Bombus terrestris*, *B. lapidarius* and *B. subterraneus*, nest underground in abandoned nests of rodents and, therefore, are protected from direct spray applications. However, other species, such as *B. pascuorum* and *B. ruderarius* in Europe, typically nest above ground under patches of grasses and vines where there is greater potential exposure to drift, or direct pesticide applications (Pouvreau, 1984; Thompson, 2001). Stingless bees and bumblebees mainly use wax to build their nests, but, unlike honey bees, they also commonly mix it with pieces of grass, leaves and various substrates (Pouvreau, 1984; Roubik, 1989), which may also be a source of exposure to contaminants.

Among solitary bees, the location of the nests as well as the material used to build them can vary considerably. Most (ca. 80%) non-*Apis* bees are soil-nesting and they dig burrows of various length usually in areas of bare ground and in soils of varying texture, the most common being sands and loams (Cane, 1991; Michener, 2007). The gregarious ground nesting species can occur in large aggregations of several 1000s of individuals in natural sites (*e.g.*, Potts and Willmer, 1998) or in man-made bee beds such as for *Nomia melanderi* (Cane, 2008). In addition, ground-nesting bees can be found along the border of fields planted with annual crops, but also in the soil within such fields (Vaissière *et al.*, 1985; Shuler *et al.*, 2005; Kim *et al.*, 2006). Therefore, dissipation rate of pesticides in soil is a key factor affecting potential exposure to these species.

The second largest group of solitary bees consists of species that nest in pre-existing cavities (mostly tunnels) in dead wood, hollow twigs and bamboo, or pithy stems such as elderberry (*Sambucus* spp.). These include most bees in the genera *Osmia* and *Megachile* (Cane *et al.*, 2007). Other species, such as carpenter bees (*Ceratina* spp., *Lithurgus* spp. and *Xylocopa* spp.) drill their nest tunnels in soft wood or the soft pith of some plant stems.

Among the "tunnel nesters", leafcutter bees (Megachilidae, especially *Megachile* spp.) use leaf pieces, as their common name suggests, to line their burrows and seal each cell once their egg has been laid on a ball of pollen and nectar. These leaf pieces are collected from a large array of plants, such as alfalfa (*Medicago sativa*) and rose bushes (*Rosa* spp.)

Leafcutter bee on blanket flower, photo by Mace Vaughan

Other bees build their nests with flower petals (*e.g.*, *Hoplitis* spp.), or plant hairs (*e.g.*, wool-carder bees such as *Anthidium manucatum* (Gibbs and Sheffields, 2009), and many mason bees, *Osmia* spp., use mud to build partitions between the different cells of their nests (*e.g.* Bosch and Kemp, 2001; Mader *et al*., 2010).

Pesticide contamination of these nest materials can occur and may ultimately present a risk, particularly in the case of contact insecticides (Waller, 1969; Johansen and Mayer, 1990). The increasing use of systemic insecticides, including those labeled for landscape use, may result in exposure to nest material for leaf-cutter bees (Vera Krischik, personal communication), especially some species of *Osmia* that chew up pieces of leaves to create walls of pulp to separate brood cells.

Immature stages

As stated previously in this chapter, honey bee worker and drone larvae are fed primarily processed food (larval jelly and bee bread). Indeed, raw, unprocessed pollen fed directly to worker and drone larvae comprises less than 5% of the total protein consumed during honey bee larval development (Haydak, 1970; Babendreier *et al*., 2004). Queen larvae receive even less directly-fed [unprocessed] pollen, as royal jelly contains only traces of pollen (Haydak, 1970).

The process by which the bee donverts stored pollen and nectar into royal jelly may result in modifications (*e.g.* degradation) of pesticide active ingredients in food stores. Also the pollen stored by honey bees in the comb undergoes a lactic fermentation to become bee bread so that many kinds of microbiological and chemical changes occur between the corbicular pollen brought in by the workers, and the stored pollen which is processed and feed to the larvae (Gilliam *et al*., 1989). However, the level of processing and degradation may be different for other bee species (Fernandes da Silva and Serrao, 2000).

Thus, the exposure to pesticides for honey bees that are fed processed pollen/nectar (*e.g.*, larval jelly and bee bread) may differ from that of solitary non-*Apis* bees whose larvae feed directly on a mass of raw pollen and nectar mixed together, or even from that of pocket-feeder bumble bees that use sequential mass provisioning. In sequential mass provisioning, a cluster of brood cells is provisioned over various timeframes.

Indeed, direct feeding on a mass of raw pollen and nectar mixed together is the rule in all solitary non-*Apis* bees as well as the social sweat bees (Halictidae). With this in mind, exposure estimates based on stored honey bee pollen that is subsequently converted to bee bread and larval jelly is unlikely to be predictive of the residues to which non-*Apis* bee brood is exposed: both through oral exposure when this mix is consumed and contact exposure as the eggs and larvae are in direct contact with the raw pollen-nectar mix (Konrad *et al*., 2008).

Foraging and mating

Among solitary bees, males are the first ones to emerge from the nest followed a few days later by females. Non-*Apis* bees vary considerably in adult size from a few mm (*e.g. Perdita* spp. in the Ne World and *Nomioides* in Europe) to the very large carpenter bees (*Xylocopa* spp.) and bumble bee queens (*Bombus* spp.) that routinely reach 3-cm long or more (Michener, 2007). Most non-*Apis* bees are smaller than honey bees, and therefore can be exposed to relatively higher doses of pesticides by contact because of the higher surface area to volume ratio of smaller species. (This has been demonstrated with intra-specific [pesticide toxicity] tests that have indicated that some smaller bees are more sensitive than larger bees at similar exposures on a unit / bee basis (Thompson and Hunt, 1999; Malone *et al*., 2000).

Peak foraging time for honey bees is generally during warm, non-overcast conditions (Riedl *et al*., 2006; Tew, 1997; Johansen and Mayer, 1990). However, this is not the case for many non-*Apis* bee species, such as bumble bees and mason bees (*Osmia* spp.*)*, which are known to forage during cool, inclement weather, as well as earlier and later in the day, and earlier and later in the season than honey bees (Thompson and Hunt, 1999; Vicens and Bosch, 2000; Bosch and Kemp, 2001; Thompson, 2001). Similarly, squash bees (*Peponapis* & *Xenoglossa* spp.) are active in the early pre-dawn hours (Sampson *et al*., 2007). In addition, males of many non-*Apis* bees often spend the night in flowers or hanging from plants, potentially leading to higher exposures (Sapir *et al*., 2005). Although, male squash bees that spend the night in closed squash blossoms may receive some level of protection from nighttime pesticide applications because the blossoms close tightly around them.

Non-*Apis* bees may also forage and even specialize on plants not readily visited by honey bees, such as the buzz-pollinated (*i.e*., activity that releases pollen that is tightly held by anthers) solanaceous tomatoes (*Lycopersicon esculentum*, Greenleaf and Kremen, 2006) and potatoes (*Solanum tuberosum*, Sanford and Hanneman, 1981), some legumes with long corolla[5] (Richards, 1987) and some ornamentals. For example, tomato and potato flowers do not produce nectar and their anthers release pollen through small pores rather than large slits. Consequently honey bees do not visit these plants, where as many non-*Apis* species do. Although, it is possible that pollen from flowers of this type could be shielded from foliar pesticide applications because of the unique plant morphology.

Honey bees are extreme generalists in that a colony will forage for nectar and pollen on a large array of plant species (polylecty). This is not so for most non-*Apis* bees, especially for the 80% or more which are solitary. These species often gather their pollen on a few species of taxonomically related plant species (oligolecty) and sometimes on a single species. For example, squash bees gather all their pollen and most of their nectar on flowers of *Cucurbita* spp. As a result, a pesticide applied to a field of squash may be well diluted in a honey bee colony whose workers are foraging from various floral resources across a wide landscape, but not for the progeny of a squash bee that foraged on that crop that day.

Another factor affecting foraging and exposure in non-*Apis* bees is the direct relationship between foraging distance and species size. While large bees, such as honey bees, bumble bees or carpenter bees (*Xylocopa* spp.), easily forage over several km from their nest (Beekman and Ratnieks, 2000; Goulson and Stout, 2001; Pasquet *et al.*, 2008), small bees may only fly a few hundred meters from their nest site (Greenleaf *et al.*, 2007). This factor potentially results in a disproportionate exposure of small bees that are attracted to blooming crops, where their limited foraging range necessitates nearby nesting, and ongoing exposure to pesticide applications throughout the growing season. In some landscapes (*e.g.*, New Jersey, USA), small bees (*e.g.*, *Halictus* and *Lasioglossum* spp.) perform a significant amount of crop pollination (Winfree *et al.*, 2007a; Winfree *et al.*, 2007b).

Methods and Models for Estimating Exposure of Bees to Pesticides

Currently, there are no globally-accepted approaches for estimating exposure of pesticides to bees for screening-level risk assessments. Participants of the Workshop reviewed current methodologies employed in the U.S. and EU, and evaluate information that can be used or

[5] Corolla: defination….

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developed to establish exposure estimates for screening-level risk assessments for both honey bees and non-*Apis* bees.

Current methods

Atkins method (Canada/US), use in North American label warning statements

Atkins *et al.* (1981) conducted laboratory contact toxicity studies and corresponding field studies with 65 pesticides. The field hazards were studied in a large number of commercial fields during bloom using crops that were highly attractive to honey bees. Based on the results of the studies, toxicity categories were developed to classify pesticides.

Based on the data developed by Atkins *et al*., the median lethal dose (LD_{50}) in micrograms of active ingredient per bee (µg a.i./bee) from the laboratory contact toxicity test can be converted to the equivalent number of pounds of chemical per acre when applied as a spray to the aerial portions of plants (for kilograms per hectare, multiply µg a.i./bee by 1.12). For example, an acute contact LD_{50} of 1 µg a.i./bee (highly toxic according to Atkins *et al.* classification scheme) would equate to an application rate of 1 pound per acre (1.12 kg a.i./ha).

EU Hazard Quotient method

In the European Union, the Hazard Quotient (HQ) approach is used as a screening-level assessment to distinguish between low and high risk of acute poisoning for foliar pesticide applications. The HQ relates the application rate of a product with laboratory oral and contact LD_{50} values.

HQ = Application rate (g a.i./ha) / Contact or Oral LD_{50} (µg a.i./bee)

Since exposure component of this expression is simply the application rate (and not measured exposure) an HQ value can be viewed as a higher-tier hazard classification and not a true risk estimation.

EPA Residue Unit Dose (T-Rex), comparison of lab contact toxicity data with residue data from T-REX

Exposure of foliar applied pesticides to bees has been estimated by U.S. EPA using the Terrestrial Residue Exposure Model (T-REX). This model is used to predict residues on food items (vegetation, seeds, insects, etc.) for birds and mammals, and is based on a nomogram developed by Hoeger and Kenaga (1972). The dermal exposure to a bee is calculated by multiplying the residue predicted for broadleaf plants/small insects by the assumed weight of a foraging honey bee (0.128 g) (Mayer and Johansen, 1990) to establish a dose per bee (ug ai/bee).

Although this method could potentially be useful for developing a screening-level exposure estimate for bees, the values developed by Hoeger and Kenaga (1972) to estimate residue values on insects are not based on residue data for insects but rather on plants or plant parts of similar size (Fletcher *et al*., 1994). Data from Hart and Thompson (Hart *et al*., 2001) indicate that the 95th percentile value for an insect residue per unit dose (RUD) is 24 mg/kg compared to 135 mg/kg for broadleaf plants (EPA's surrogate for small insects) which is approximately 6 fold higher. Data from additional studies (Fischer and Bowers, 1997; Brewer *et al*., 1997) also suggest that the insect residue estimates developed by Hoeger and Kenaga (1972) are greatly overestimated.

ICPBR proposal for seed treatment or soil applied systemic compounds

The main route of exposure of bees to residues from systemic seed treatments and soil applications is through the translocation of the compound into nectar and pollen. Alix *et al.* (2009a) have compiled and analyzed available data on measured residue levels in different plant parts. Residue levels in plant parts were measured after treatment with systemic insecticides for the purpose of developing Tier 1 exposure assessments.

The compiled residue data base considered residues values as close as possible to bloom. Based on their analysis, a default maximum residue value of 1 mg a.i./kg plant matrix has been proposed as a worst-case, peak value for the screening-level exposure estimate for systemic compounds used as seed treatments or applied to soil (Alix *et al*., 2009a, Alix and

Lewis, 2010). In the event the Tier 1 risk assessment based on this worst-case estimate indicates a potential risk, actual measured residues from higher-tier studies can be used for a refined risk assessment. If there is a need to transform the Tier 1 predicted concentrations in pollen and nectar into predicted doses to honey bees, it is recommended to follow the proposals as outlined by ICPBR (Alix *et al.*, 2009a), which uses pollen and nectar consumption rates by different casts of honey bees (Rortais *et al.*, 2005). The published consumption rates are provided later in this chapter (see Predicted Dietary Exposure to Foliar Applied Products).

Physical and chemical properties of pesticide active ingredients that affect exposure

The physicochemical properties of the pesticide active ingredient determine its fate in soil and in hive matrices which can affect the exposure of the various life stages of the honey bee to these chemicals.

1) Fate in soil – systemic products

Systemic products applied to soil can be taken up by the plant and translocated into plant foliage, floral nectar and pollen. Persistent systemic products that remain in the soil for over one year could potentially be translocated into the nectar and pollen of rotational crops planted in succeeding years. The dissipation time or DT_{50} is used to characterize the persistence of pesticides in soil.

Physicochemical properties of the pesticide active ingredient that can affect persistence in soil include water solubility, the octanol-water partition coefficient (K_{ow}), dissociation constant (K_a), the soil adsorption coefficient (K_d) and the organic carbon partition coefficient (K_{oc}). Pesticides with high water solubility and low K_{oc} (*e.g.*, < 50) values have a higher potential for mobility, do not strongly adsorb to soil particles and can be prone to leaching depending on soil conditions, weather and persistence of the compound. The Log of the K_{ow} (log K_{ow} or log P) is the measure of a chemical's propensity to bioaccumulate. Pesticides with a high log P (*e.g.*, > 3) usually have low water solubility and are not highly mobile in soil. The log of the dissociation constant (pK_a) is a measure of the extent to which a substance ionizes in equilibrium with water. The pKa of a pesticide indicates the ratio of the forms (ionized or undissociated) in which the chemical will exist in environments of various pH values, and extent of its potential involvement in ion-exchange binding processes in soils or sediments. The form of a pesticide (anion or cation) can influence its mobility and hence persistence in soil. Soil type and meteorology (amount of rainfall, temperature) can also influence the persistence of a pesticide in soil.

Specific criteria to classify compounds as being persistent in soil have been identified by the EU (EEC, 2006) and other regulatory agencies to trigger the requirement of rotational crop residue studies (used to inform human health risk assessment). It has been proposed that similar criteria be used to require assessment for the risk of residues in pollen and nectar for succeeding crops (Alix and Lewis, 2010).

2) Fate in hive matrices – systemic and non-systemic products

Physicochemical properties including water solubility, log P, and the pK_a can influence fate of the active ingredient in the hive. Compounds with a high log P that are hydrophobic (*i.e.*, tending not to be soluble in water) may accumulate in wax, pollen, and bee bread which contain lipids. Compounds with a high solubility in water (hydrophilic) can partition to nectar and honey which contain water. If the compound dissociates, the dissociation constant may be used to indicate fate in acidic matrices such as honey.

Information needed to develop refined predictive exposure models

As stated above, there are no defined predictive models currently used for estimating exposure levels in bees or bee matrices to compare with hazard data for a screening-level ecological risk assessment. The current procedures used by the EU (Hazard Quotient approach), Canada and U.S. (based on Atkins data) which employs conservative values for potential exposure, have been effective in screening-out compounds that have low potential risk to adult worker bees from foliar-applied products. However, for crop protection products where potential risk cannot be excluded based on current Tier 1 screening analysis, the current method to refine assessments consists of higher-tier effects or exposure assessment studies (*e.g.*, EPA Tier 2 foliar residue study, EPPO tunnel test).

Optimally, there should be methods to predict residue levels in relevant matrices (*e.g.*, bees, pollen, nectar). These predicted exposure concentrations could then be used to compare with laboratory toxicity data, such as acute contact LD_{50} values for adult bees, and acute and chronic dietary toxicity data for adult bees and larvae to estimate risk to both foraging bees and other casts and life-stages in the hive, including larvae.

Predicted Contact Exposure for Foliar-Applied Products

For foliar-applied products, the prediction of residues on foraging bees due to contact exposure (*i.e.*, direct spray on foraging bees or bees contacting residues post-spray) can be estimated. The U.S. EPA has proposed using predicted concentrations in insects based on estimates in their T-REX wildlife exposure model. However, as noted above, there are some inherent uncertainties with using this approach. In this approach, values from T-REX Version 1.4.1, which relies on residue estimations developed by Hoeger and Kenaga (1972) for plants, fruits, and seeds, would be used as surrogate data to estimate contact exposure for insects. However, actual field residue data are available for honey bees (Koch and Weißer, 1997) and a variety of flying, soil-dwelling and leaf-dwelling arthropods (Schabacker *et al.*, 2005) that can be used for estimating contact exposure to bees. In a multi-year study by Koch and Weißer (1997), the fluorescent tracer sodium fluorescein was applied to flowering apple orchards or flowering *Phacelia* fields while honey bees were actively foraging, to determine contact doses in individual honey bees. After applications of 20 g sodium fluorescein/ha, doses in honey bees ranged from 1.62 to 20.84 ng/bee, and 6.34 to 35.77 ng/bee for honey bees foraging in apples and *Phacelia*, respectively. If the maximum detected residue in this study (35.77 ng/bee after an application of 20 g/ha) was used as a point estimate for a screening-level exposure assessment, a **Predicted Environmental Dose due to contact exposure (PEDc) in adult honey bees after an application of 1 kg/ha (1000 g/ha) would be 1789 ng/bee or 1.79 μg/bee.** The assumption here is that there will be a linear relationship between application rate and contact dose of foraging bees, which is an area of uncertainty.

In the report by Schabacker *et al.* (2005), maximum residues in flying, ground-dwelling and foliage-dwelling arthropods from a number of field trials were compiled and residue unit doses (RUDs) were calculated. The mean and 90th percentile RUDs in mg/Kg after application of pesticides at a rate of 1 kg as/ha are summarized in the following table:

Predicted Concentrations (in mg/Kg) After Foliar Application of 1 kg/ha*

Arthropod classification	**Mean Predicted Concentration in mg/kg**	**90th Percentile Predicted Concentration in mg/kg**
Flying insects	1.4	6.6
Ground-dwellers (orchard/vines, grasslands, late growth stages of leafy crops and cereals (insecticides and fungicides))	3.6	9.8
Ground-dwellers (orchard/vines (herbicides), early growth stages of leafy crops and cereals (all pesticides)	6.7	15.6
Leaf-dwellers	9.5	47.8

*Data from Schabacker *et al.* (2005)

When residue data for flying insects are used to develop a screening-level point estimate for contact exposure of foraging bees, a **90th percentile PEDc after an application of 1 kg a.i./ha is calculated to be 0.84 µg/bee.** This is derived by multiplying the 90th percentile concentration in flying insects (6.6 mg/kg) by the weight of an adult foraging honey bee (128 mg) (Mayer and Johansen, 1990). This point estimate (0.84 µg/bee) is close to the exposure value calculated using the data of Koch and Weißer (**1.79 µg/bee**), and is consistent with the data developed by Atkins *et al.* (1981), where a dose of 1 µg/bee represents an application rate of 1 lb a.i./A. Therefore, according to the Atkins method, an application of 1 kg a.i./ha is equivalent to an exposure value of **0.89 µg/bee**.

Based on the above information, a worst-case estimate predicted exposure dose for contact (PEDc) to honey bees after an application of 1 kg a.i./ha is 1.79 ug/bee.

To evaluate the sensitivity of the proposed point estimate of exposure for honey bees (*i.e.*, **1.79 µg/bee after an application of 1 kg a.i./ha**) a generic data set of contact LD_{50} values and use rates can be used to calculate Hazard Quotients, Toxicity / Exposure Ratios (TER = LD_{50} in µg a.i./bee / PEDc in µg a.i./bee) and Risk Quotients (RQ = PEDc / LD_{50}). Using a generic data set with an application rate of 100 g a.i./ha, the corresponding HQ, TER and RQ values are summarized in the following table.

Table X. Comparison of Hazard Quotient (HQ), Toxicity/Exposure Ratios (TER) and Risk Quotients (RQ) assuming a predicted contact exposure dose (PEDc) of 1.79 µg a.i./bee after an application of 1 kg a.i./ha.

Use Rate	PEDc	Contact LD50	HQ	TER	RQ
0.1 kg / ha	0.179 µg / bee	1 µg / bee	100	5.6	0.18
0.1 kg / ha	0.179 µg / bee	2 µg / bee	**50**	**11**	**0.09**
0.1 kg / ha	0.179 µg / bee	5 µg / bee	20	28	0.036
0.1 kg / ha	0.179 µg / bee	20 µg / bee	5	112	0.009
0.1 kg / ha	0.179 µg / bee	50 µg / bee	2	279	0.0036
0.1 kg / ha	0.179 µg / bee	100 µg/ bee	1	559	0.0018

According to Annex VI of the EU Uniform Principals, a TER of ≥ 10, designed to cover inter species variability, typically indicates acceptable risk for terrestrial organisms, and has been recommended as an appropriate assessment factor for oral exposure to systemic insecticides by ICPBR (Alix *et al*., 2009a,b; Alix and Lewis, 2010). U.S. EPA on the other hand uses a level of concern (LOC) RQ of 0.1 for non-listed threatened or endangered aquatic or avian species. Based on this analysis, the screening-level risk assessment based on a PEDc of 0.179 µg/bee is in-line with the current EU screening HQ of 50.

Although the published field trail data (Koch and Weißer, 1997) for residues on honey bees are most appropriate for developing exposure estimates for honey bees, it might be more appropriate to use the data for leaf-dwelling and soil-dwelling arthropods from the data developed by Schabacker *et al.* (2005) to address exposure to leaf-dwelling and soil-nesting non-*Apis* bee species, respectively. Therefore, for the initial, conservative point estimate of contact exposure, the 90th percentile predicted concentration for leaf-dwelling arthropods (47.8 mg/kg), can be used to develop a PEDc for leaf-dwelling species, while the 90th percentile predicted concentration for soil-dwelling arthropods (15.6 mg/kg) can be used to develop a PEDc for soil-nesting species. However, in order to complete this analysis and develop recommend PEDc values for leaf-dwelling and soil-nesting non-*Apis* bees, focal species need to be identified. The leaf-dwelling species, leafcutter bee (*e.g.*, *Megachile rotundata*) is recommended as surface dwelling non-*Apis* reference species, while a bumble bee (*Bombus* spp.), which typically nest on or under ground, or mason bee (*Osmia* spp.), which collect mud for nest construction, are recommended for soil-nesting (gregarious) focal species. Ideally, ground-nesting solitary bees, such as sweat bees (*e.g.*, *Halictus* or *Lasioglossum* spp.), squash bees (*Peponapis* or *Xenoglossa* spp.), or alkali bees (*e.g.*, *Nomia melanderi*) also would be considered as representative soil-nesting species, for these insects dig nests underground. However, at least in North America, only *Nomia melanderi* is currently managed successfully at a larger scale. With the identification of focal species, the typical body weights of the species can be used convert predicted exposure concentrations in mg/kg to PEDc values in µg/bee for direct comparison to laboratory toxicity data.

Prior to adopting this proposed methodology into a formal regulatory assessment paradigm for bees, the method should be used to calculate toxicity/exposure ratios for some representative compounds to ensure that the exposure assessment methodology is sensitive enough to predict an acute risk to compounds that are highly toxic to non-*Apis* bees (*e.g.*, pyrethroid insecticides), while not predicting a high risk for compounds that are known to have low inherent toxicity and present a low risk to non-*Apis* bees.

Predicted Dietary Exposure for Foliar Applied Products

For assessing acute or chronic dietary risk to adults or larvae, predicted concentrations in relevant food items (*e.g.*, pollen, nectar, beebread, honey, and larval jelly) should be used as the dietary exposure estimate. Currently, models to predict residues in these items from foliar applied pesticide products do not exist. Although the results from survey-style analysis indicate that agricultural pesticides are entering managed honey bee colonies through contaminated pollen (Chauzat *et al*., 2010; Mullin *et al*., 2010), there are limited published data from controlled studies which relate foliar application rates to measured pesticide levels in pollen and nectar of in any processed food.

In a study by Choudhary and Sharma (2008) residues of three foliar applied pesticides were determined in nectar and pollen following applications to blooming mustard. Pesticides evaluated in this two-year study were endosulfan, lamda-cyhalothrin, and spiromesifen. Mean measured residues in pollen and nectar, and extrapolated predicted concentrations after application of 1 kg a.i./ha are summarized in the following table.

Table X. Day 0 Measured Concentrations of Three Foliar Applied Pesticides in Pollen and Nectar after Application to Flowering Mustard[a]

Compound	Application rate (g a.i./ha)	Mean Measured Residues Nectar[b] (mg/kg)	Mean Measured Residues Pollen[b] (mg/kg)	Mean Extrapolated Nectar Residues (mg/kg) After Application of 1 kg/ha	Mean Extrapolated Pollen Residues (mg/kg) After Application of 1 kg/ha
Endosulfan	525	1.725 ± 0.031	2.126 ± 0.088	3.15	3.99
		1.583 ± 0.006	2.068 ± 0.048		
Lamda-cyhalothrin	75	0.858 ± 0.038	1.607 ± 0.004	10.6	21.2
		0.728 ± 0.022	1.577 ± 0.018		
Spiromesifen	225	1.541 ± 0.078	2.003 ± 0.040	6.54	8.45
		1.401 ± 0.016	1.799 ± 0.033		

[a]Data from Choudhary and Sharma (2008)
[b]Mean measured residues from two successive application and sampling years

In a study by Wallner (2009), residues of the fungicides boscalid and prothioconazole were determined in pollen and nectar samples from foraging bees following applications to oil seed rape (canola). Mean measured residues in pollen and nectar, and predicted concentrations after application of 1 kg a.i./ha are summarized in the following table.

Table X. Day 0 Measured Concentrations of Two Foliar Applied Fungicides in Pollen and Nectar Collected from Honey Bees after Application to Flowering Oil Seed Rape[a]

Compound	Application Rate (g a.i./ha)	Mean Measured Residues Nectar (mg/kg)	Mean Measured Residues Pollen (mg/kg)	Mean Predicted Nectar Residues (mg/kg) After Application of 1 kg/ha	Mean Predicted Pollen Residues (mg/kg) After Application of 1 kg/ha
Boscalid	500	1.43	26.2[b]	2.86	52.4
Prothioconazole	250	0.69	nd (LOQ = 0.001)	2.76	-----

[a]Data from Wallner (2009)
[b]Concentrations 1 day after treatment, which were higher than day-0 values

Finally, in a study by Dinter *et al.* (2009), concentrations of the insecticide chlorantraniliprole in pollen and nectar collected from foraging bees following applications to *Phacelia* in a semi-field study were determined. The maximum concentrations in pollen and nectar 1-day after treatment is summarized in the following table.

Table X. Day 1 Measured Concentrations of Chlorantraniliprole in Pollen and Nectar Collected from Honey Bees after Application to Flowering *Phacelia*[a]

Compound	Application Rate (g a.i./ha)	Maximum Measured Residues Nectar (mg/kg)	Maximum Measured Residues Pollen (mg/kg)	Maximum Predicted Nectar Residues (mg/kg) After Application of 1 kg/ha	Maximum Predicted Pollen Residues (mg/kg) After Application of 1 kg/ha
Chlorantraniliprole	60	0.033	2.60	0.55	43.3

It is difficult to draw any firm conclusions based on these limited published data. For instance, there is not a linear relationship between application rate and measured concentration in pollen and nectar across the different compounds. Therefore, the predicted concentrations after applications of 1 kg/ha may be greatly exaggerated for some compounds. It is likely that the variation in residue levels seen between these studies is a result of different factors such as sampling, extraction methods, fate properties of the different compounds, or product formulation, etc.

Although limited published data are available for maximum residue levels in nectar and pollen after controlled applications of foliar products, there is likely a significant amount of data that have been developed by pesticide manufacturers for individual products. Therefore, the participants of the Workshop proposed that nectar and pollen residue data from semi-field exposure studies conducted according to EPPO guidelines be compiled and analyzed. These data should represent maximum residues in bee food items in a bee-attractive crop, and developing models around these data would likely provide realistic, worst-case predicted residues for a screening-level risk assessment.

Once these data are compiled, a conservative estimate for residues on/in pollen and nectar (*e.g.*, 90th percentile RUDs) can be used to calculate TER or RQ values. These screening-level predicted values would represent a conservative estimate of dietary exposure for honey bees from foliar application of pesticide products. For a dietary risk assessment, the predicted concentration of residues in food items can be directly compared with the results from dietary toxicity studies with adult bees and bee larvae, if the results from the studies are expressed as exposure concentrations (*i.e.*, LC_{50}, NOEC). However, if the toxicity results are expressed as a dose (*i.e.*, LD_{50} in μg/bee), the predicted dose can be calculated based on predicted concentrations on food items and consumption rates by different casts of bees. Published honey bee consumption data, based on complete live-stages, has been reported by Rortais *et al.* (2005), and are summarized below:

Nectar foragers – 224 – 898.8 mg sugar
Pollen foragers – 72.8 – 109.2 mg sugar
Nurse bees – 65 mg pollen
Worker larvae – 59.4 mg sugar + 5.4 mg pollen
Drone larvae – 98.2 mg sugar

The following daily consumption rates for the different honey bee casts were calculated by Thompson (2007):

Nectar foragers – 32 – 128.4 mg sugar/bee/day
Pollen foragers – 10.4 – 15.6 mg sugar/bee/day
Nurse bees – 6.5 mg pollen/bee/day
Worker larvae – 11.9 mg sugar + 1.1 mg pollen/bee/day
Drone larvae – 15.1 mg sugar/bee/day

For dietary risk assessments, it will be important to choose the appropriate consumption rate data to evaluate acute and chronic risks, *i.e.*, the daily consumption rate should be compared with acute oral toxicity data to estimate acute risks, while life-stage consumption data should be compared with chronic toxicity data to estimate chronic risk.

Predicted Exposure for Soil and Seed Treatment Systemic Compounds

For soil-applied or seed treatment systemic products, the current ICPBR proposal recommends using a default maximum exposure value of **1 mg/kg for pollen and nectar**, which is based on analysis of existing residue data (Alix *et al*., 2009a). Currently, the number of standardized exposure studies, evaluating residues in pollen and nectar for systemic pesticides is limited to a few compounds for the same class of chemistry (*i.e.*, neonicotinoids) (Alix *et al*., 2009b). Therefore, there may not be enough data to develop a predictive exposure model applicable to all soil-applied or seed treatment systemic compounds. In the case of systemic compounds, it appears that residues in pollen and nectar are not only influenced by the physical and chemical properties of the compound (*e.g.*, K_{oc}, soil DT_{50}, K_d, pollen and nectar uptake and dissipation), but also be soil properties, crop, weather, and application timing versus time of bloom. Therefore, as pollen and nectar residue data for other classes of systemic compounds are developed, the above mentioned variables should be considered. As more residue data are developed for systemic compounds (both neonicotinic and other classes), the concept of developing a predictive screening-level exposure model should be explored further. **In the interim, the default value of 1 mg/kg is recommended as the point estimate for exposure in Tier 1 risk assessment for dietary exposure to systemic compounds, as it represents a current worst-case estimate of residues in matrices that are consumed by bees (*i.e.*, pollen and nectar). However, as more data is developed for systemic compounds, this value should be re-evaluated to ensure that 1 mg/kg is conservative enough for a screening-level risk assessment.**

Predicted Exposure for Tree-Injected Compounds

Certain insecticides can be directly injected into tree trunks for control of wood boring insects. The chemical enters the xylem and is systemically transported to all parts of the tree including nectar (if produced) and pollen, and potentially propolis, which is not consumed, but used by bees in the construction and maintenance of nests and hives. There is a scarcity of data are available on residues of pesticides detected in nectar, pollen and propolis from tree-injections. It is unclear if the residue value of **1 mg/kg**, as proposed by ICPBR for soil and seed treatments, is appropriate as a maximum default residue for a screening-level risk assessments for tree injection. Until more information on potential exposure from this application method is developed, it is recommended that pollen, nectar and propolis (if applicable) samples be collected from treated trees and analyzed for residue content to determine appropriate exposure values which can be used in a risk assessment.

Measuring Pesticides in Matrices Relevant for Assessing Exposure to Bees

When quantification of pesticide residues in bees or bee food is required to refine an exposure assessment, it must be determined whether the goal is to assess exposure of adult forager bees or other members of the hive (queen, nurse bees, drones and larvae). To determine exposure of foragers from foliar applications, analysis of bees collected from the sprayed crop can be conducted. For exposure of forager bees from oral sources, samples of nectar and pollen can be collected by hand from flowers or from foraging bees on the crop. Bees may be sampled by drawing nectar from the honey stomach and pollen can be removed from the pollen baskets. Whether it is more time and cost effective to use bees to collect samples or doing it by hand sampling is dependent on the type of crop flower being sampled.

Where collection of nectar from the target crop is possible by hand, this can be done by inserting a micro capillary tube or pipette into the nectary and extracting the nectar. Collection of pollen by hand can be done by shaking flowers or using scissors to remove anthers followed by separation of the pollen from the anthers either in the field or after transportation to a laboratory. Flowers from several crops have very little, if any, nectar and pollen, making hand collection impractical. In these instances, bees can be used to collect the samples. Collection of nectar using bees can be done by vacuuming the bees that are actively foraging on flowers in the crop of interest. However, vacuuming bees from trees may be impractical depending upon the height of the trees and the limited amount of bees on a tree at one time. Another way to sample bees is by collecting them at the hive entrance. However, verification that the bees came from the crop of interest should be done. This can be accomplished by identifying pollen brought back to the hive or by confining the bees during the exposure portion of the study using a semi-field study design. Pollen samples should be characterized to ensure that the bees actually foraged on the target crop during field studies. To obtain the nectar sample, honey stomachs can be dissected from the bee and contents drained into a vial or the honey stomachs can be pierced with a syringe or micropipette and the nectar can be extracted. Pollen can be obtained from bees collected from flowers or at the hive entrance by removing the pollen from the pollen baskets. Pollen samples can also be collected in pollen traps attached to the hive entrance. If either pollen or nectar cannot be efficiently collected in large enough quantities for residue analysis, whole flower samples could also be analyzed for possible use as a surrogate (pending further collection and analysis of these data).

For potential exposure to residues in stored pollen, nectar and larval jelly, samples from the hive can be drawn. Stored pollen can be sampled by identifying frames where fresh pollen is being stored and removing this pollen with a spatula from individual cells. Adding an empty comb can ensure that the pollen and nectar is freshly collected. Nectar can be sampled by identifying the frame where fresh nectar is being stored, removing the frame from the hive, and shaking the frame into a large pan to release the nectar. The released nectar can then be transferred to a vial using a pipette, or pouring if the volume allows. Alternatively, fresh nectar can be identified and extracted from individual cells using a syringe or pipette and transferred to a vial. Larval jelly can be identified on the frames and either extracted from the cells with a capillary tube or pipette, or by removing the larvae and scooping out the jelly with a spatula and transferring it to a vial.

All samples collected in the field should be kept on ice until received by the analytical laboratory. At the laboratory, samples should be stored frozen (-20ºC) and protected from light until analysis. Experience shows that plastic storage containers should be used with caution because some pesticides can sorb to plastic. Standardized procedures for sampling, including appropriate storage and transport, should be established in order to avoid contamination, and provide adequate sample size. Specific, statistically valid, plans for sample size and number also should be established in the study protocol. Dedicated coolers, chain of custody, records of transport and storage conditions and other appropriate Good Laboratory Practice procedures should be used and documented to insure sample integrity. The quantity of samples needed for analysis of pesticide residues should be determined prior to sampling and might vary based on limits of detection and limits of quantification for each pesticide in the individual matrices. Use of spiked samples, to accompany samples collected from the field, can be used to assure sample integrity. Analytical methods also need to be properly validated to insure that extraction methods are adequate and the residues of interest are accurately identified.

At the present time it is recommended that collection of nectar and pollen directly from the flowers, or collecting and removing pollen and nectar from foraging bees would be the most conservative and most relevant estimates of exposure for bees outside the hive. For larvae, nurse bees, drones and the queen in the hive, sampling freshly deposited nectar and pollen from the combs would be the most conservative dietary exposure estimate, considering additional processing of these materials by bees may result in lower concentrations in other hive food sources. To further refine these estimates, data on the comparative residue levels in flowers, nectar, pollen and hive products (such as stored pollen, nectar, honey, larval jelly, and beebread) can to be generated to determine worst-case oral exposure estimates for either foraging bees and hive bees

Mircopipetting nectar samples; photo by Mike Beevers

Hand-collecting pollen by removing flower anthers, photo by Mike Beevers

Higher-Tier Studies to Assess Exposure of Pesticides to Bees

Higher-tier study to evaluate contact exposure to honey bees

In the U.S., if a compound is classified as being toxic to honey bees by contact exposure (*i.e.*, LD_{50} < 11 µg/bee), a Tier 2 contact residue study is required to determine appropriate label warning statements for pollinators. In this study, a bee attractive plant (typically alfalfa) is sprayed with formulated product at the maximum application rate. Groups of worker bees are caged over the treated crop at various time points after application (typically, 0, 4, 8 and 24 hours), to evaluate the bioavailablity and persistence of pesticide residue. These data are used to determine the length of time between application and when bees can be safely exposed to a treated crop. From this test, a residual toxicity time is established indicating where the pesticide residue is lethal to 25% of the test organisms, refereed to as the RT_{25} .

Higher-tier exposure studies using honey bee colonies

Since it is not economical to conduct exposure studies in every crop, realistic worst case model crops should be used for assessing exposure of bees under field-relevant use conditions in semi-field and field trials.

Choosing a realistic worst case model crops should include the following considerations:

- attractive to bees
- provides both nectar and pollen
- provides sufficient flower density and sufficient duration of flowering

EPPO PP 1/170 (OEPP / EPPO, 2001) proposes *Phacelia*, oilseed rape (canola), and mustard. Buckwheat (*Fagopyrum esculetum*) may also be used. Application parameters (*i.e.*, rate, interval, formulation) used in any higher-tier study should be those that are expected to produce the greatest potential exposure that is prescribed by the product label being assessed.

For a worst-case assessment of exposure, semi-field or tunnel studies can be conducted. In these studies, colonies are placed within a tent or mesh tunnel and exposed to the treated crop during or immediately after application. Using a highly bee-attractive crop would simulate a worst-case exposure to residues in pollen and nectar. Because of the controlled nature of semi-field studies for foliar-applied products, the location of the study is not as important as is for a field study. Therefore, the semi-field derived residue data should be useful globally for an exposure assessment, assuming that maximum application rates are assessed. However, in some instances, soil type and weather can influence nectar production. Therefore, optimal conditions for growing the treated crop should be followed.

Honey bee semi-field study with *Phacelia*. Photo provided by BASF

Studies to evaluate exposure from seed treatment and soil applications of systemic compounds

Regarding seed treatments and soil applications with systemic compounds, specific semi-field or field studies can be designed to measure residues in nectar and pollen in order to refine a screening-level risk assessment for systemic compounds. If the purpose of the study is to measure residue data only, the actual crop of interest should be used.

If higher tier studies are conducted and the aim is to concurrently assess residues and potential effects, preferably a crop with the highest application rate and highest attractiveness to bees should be used. If the target crop is not feasible to conduct semi-field or field studies, the use of a surrogate crop is recommended but must be scientifically justified (*e.g.*, supported by plant metabolism data, measured residue levels in nectar and pollen, *etc.*). Data on the uptake and decline of pesticide residues in pollen and nectar after systemic pesticide applications to the test crop should be evaluated prior to initiating field testing with honey bees. (Certain residue chemistry information, typically used for human health assessments may be useful in these cases.) In reviews of reports for two compounds submitted to the State of California (Bireley, 2008; Omer, 2008; Papathakis, 2008; Bireley, 2009), leaf residues in treated perennial shrubs and trees treated with imidacloprid were initially low. Residue levels were below the limit of detection for several weeks after application, but increased to levels above 10 ppm over the next several months in some instances. In addition, in some plants, leaf residues had not appreciably declined 540 days after treatment. Regardless of the timing of application, it is important that the analysis phase of field studies include sampling of the most important bee-relevant matrices (*i.e.*, pollen, nectar) during plant bloom.

Field treatments for honey bee colonies, spiked sucrose and spiked pollen

For evaluating the distribution of a pesticide throughout a hive, sucrose, pollen or protein (pollen substitute) supplements spiked with the proposed test compound (*e.g.* pesticide active ingredient) should be considered as a potential method of exposure in semi-field and field tests. Spiked pollen, protein (pollen substitute), or sucrose can also be utilized in laboratory and field testing to ensure and accurately quantify exposure to the hive.

When using spiked sucrose solution as the route of exposure for three or more days, a protein supplement is recommended to ensure effects observed are due to treatments and not insufficient nutrition. If exposure to the compound is expected to be through pollen collection and feeding, spiked protein can be fed to the test bees. An alternative is to collect and homogenize pollen from a pollen trap, spike the pollen samples with the compound being evaluated, and pressing the spiked pollen into empty combs. However, for some lipophillic compounds, pressing the pollen into a comb could end up extracting the compound if it partitions to the wax. An alternative would be to prepare a pollen cake on which the bees can forage. Also, certain pollens should be avoided because they may contain contaminants such as flavonoids that are toxic to bees. In addition, the pollen used should be pesticide free. Finally, the protein content of some pollen and differences in preference may negatively impact feeding. In some cases, researchers have used spiked protein supplements. One recommendation is to provide a 500 gram protein supplement to the colony each week during a brood cycle (*e.g.*, 21 days). Palatability and toxicity of the test compound may result in the need to alter the size of the supplement. A pollen trap may be used to significantly reduce the quantity of pollen that foraging bees bring into the hive (field studies), thus, encouraging consumption of the spiked protein supplement. A local sucrose feeder may also be used to reduce long distance foraging.

An advantage of using spiked protein supplements is that treated crops are not required and the field size the hives are placed near for forage is not relevant as long as there is adequate forage for the number of hives. In these studies, pollen traps can be used to reduce any extraneous pollen from entering the hive. Spiked protein supplements ensure that the hives are exposed to the test substance. Since the protein supplement is not specific to a particular crop, exposure is applicable to any plant where pollen is a food source.

Spiked samples may be used to validate proper handling of residue samples during collection, handling, shipping, and processing. These spikes samples should be of a similar quantity as the field samples being collected and contain the target compound at a known concentration. The samples are placed with the field samples at the time the field samples are collected. The concentration of the spiked sample should be within +/- 20% of the original concentration. These results validate that that the field handling is appropriate and the results from the field samples accurately represent actual field residues.

Health of honey bee colonies can influence exposure

In typical managed colonies, pests and pathogens are present in amounts not necessarily found in simulated laboratory and field study environment. Honey bee pathogens such as *Nosema* (Fries *et al*., 2006; Chauzat *et al*. 2007) and various bee viruses (Chen *et al*., 2006; Ribière *et al*. 2007; Chen *et al*., 2011) are commonly present in managed honey bee colonies. When colonies are subjected to changes caused by pesticide exposure, the pathogen loads can change in honey bees (Alaux *et al*.; 2010, Pettis *et. al*.; 2010). The pathogen loads can influence biological and behavioral traits of honey bees. The behavior of diseased honey bees is modified as they tend to forage earlier in their life cycle (Ribière *et al*.; 2008). Diseased individuals are often less vigorous foragers. This leads to less overall foraging activity and consequently a lesser pesticide exposure. It is often observed that the stronger colonies (*i.e*., healthier) are the most affected by poisonings, because they have more active foragers. Colonies used for testing should be healthy colonies, with minimal levels of pests and pathogens, as these can influence foraging behavior.

Higher Tier studies with non-*Apis* bee species

If a screening-level risk assessment does not indicate a presumption of low risk to non-*Apis* bee species, exposure can be evaluated using higher-tier studies. In many cases, exposure assessments for honey bee workers may address potential exposure for non-*Apis* bees (*e.g.* direct spray, systemic compounds in nectar and pollen, and spray drift). However, in some cases, non-*Apis* bees face unique exposure pathways not addressed by exposure assessments for honey bees. (see section of this chapter on Potential Routes of Exposure for Non-*Apis* Bees Species). A brief discussion regarding alfalfa leaf-cutter bees, and mason bees provides an example.

Alfalfa Leaf-Cutter Bees: contamination of nesting materials

Alfalfa leaf-cutter bees (*Megachile rotundata*) and other species of *Megachile* and *Osmia* will collect leaf pieces from a variety of plants to either wrap or build partitions between their brood cells. Common examples of plants used by these non-Apis species include species in the Rosaceae such as rose (*Rosa* spp.) and snow berry (*Symphoricarpos* spp.), bindweed (Convolvulaceae), buckwheat (*Fagopyrum esculentum*), honeysuckle (*Lonicera* spp.), wild grape (*Vitis* spp.), and wild senna (*Cassia hebecarpa*) (Mader *et al*., 2010). Alfalfa leaf-cutter bees deployed for alfalfa pollination may use pieces of alfalfa leaf collected from the very fields in which they are foraging, but often prefer buckwheat. In either case (*i.e*., wild growing plants in the surrounding landscape, or the crop targeted for pollination) there is a potential for exposure from direct application to the crop or drift to adjacent plants.

In the case of the alfalfa leaf-cutter bee used for alfalfa pollination, it is critical to understand the level of exposure from contaminated leaf pieces and, ultimately, the toxicity of this exposure (see Chapter **7** on Laboratory Testing Approaches to assess effects of pesticides for details). One possible approach would be to use a modification of U.S. EPA's guidelines for assessing the toxicity of pesticides on foliage, where alfalfa is sprayed and then brought into a laboratory at various post-application time points, and allowing bees to forage on the foliage. Another approach would be to use a semi-field or field stud design as described below:

Field or semi-field studies

1. Deploy leaf-cutter bees in closable/sealable shelters in an alfalfa field 10 days prior to pesticide application (see Appendix ??? or Lab Chapter (pp ??) on lab handling of *Megachile* and advice on incubation to adjust timing properly).

Observation tunnel-nests for the bees can be constructed to facilitate monitoring by boring a 0.6 cm (¼-inch) holes into one side of a wood plank, and covering the holes with clear acetate. Such nests should be covered with a removable opaque cover to increase nest site attractiveness. The opaque cover can be removed temporarily in order to make notations on the acetate. See also Abbott *et al.* (2008).

2. During the active nesting period, close the shelter at night to prevent foraging in the glass house, cage or field the next day. With the nest shelter closed, carefully enter it and note the constructed cells (pre-treatment) in the observation tunnels. Keeping the shelter closed, pesticides can be applied to the field adjacent (at least 200 m radius) around shelter.

3. After appropriate time has elapsed (depending upon study goals and active ingredient being used), open the shelter to allow bees to forage, build, and provision the cells.

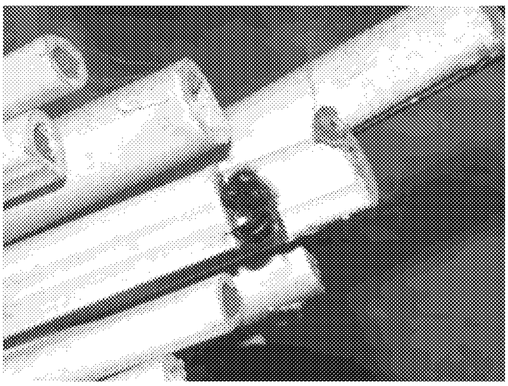
4. Note new cells created in the observation nests.

5. Newly constructed cells can be monitored for development: Eggs will hatch in ca. 15 days at 15.6 °C down to 1-to-2 days at 35 °C. Prior to egg hatching, cells may also be dissected to separate leaf pieces from cell contents (bee bread and egg) to assess:
    a. Pesticide residues in the pollen-nectar mixture (pollen ball), and
    b. Pesticide residues on leaf pieces.

6. At 15, 20, and 25+ days, cells can be sampled for presence of pesticide residues in the pollen ball, monitored for larval mortality, etc. Full development from egg hatching to adult emergence takes 35 days at 15.6 °C, but only 11 days at 35 °C.

Contamination of nesting materials: mud

Mason bees (*e.g. Osmia cornifrons, O. cornuta, O. lignaria,* or *O. rufa*) collect mud to build partitions between their brood cells (Bosch and Kemp 2001; Mader *et al.* 2010). To assess the potential level of exposure from contaminated mud, the following protocol may be used.



Mason bee. Photo by Mace Vaughan

Semi-field studies

1. Plant enclosed shelter (6 m by 2.5 m or larger) with Phacelia (*Phacelia tanacetifolia*), sweetclover (*Melilotus* spp.), or other favored forage plant. (Note: In this case, it is also possible to look for methods to use an artificial nectar or pollen feeder).

2. Deploy incubated *Osmia* spp. cocoons as loose cells or natal tubes in the enclosure at least 15 days prior to pesticide application (see Bosch and Kemp, 2001; Mader *et al*. 2010 for management advice).

   Provided the bees have undergone appropriate diapause (generally 100 to 200 days at 1.7 to 4.4 °C.), bees will begin emerging 5 to 10 of days after initiating incubation at temperatures of at least 21°C. More rapid emergence can be stimulated by incubating cocoons at 29 °C, until all bees have emerged.

   Note that male emergence precedes female emergence, often by several days, and nesting typically will not begin until one to two days after mating (which usually occurs on the day of female emergence).

3. Provide a source of wet mud with high clay content in a 1 m wide shallow pan or tray. Water this tray on a daily basis from below in order not to wash pesticide from surface. Ensure that the moisture level is not excessive leading to drowning.

4. Use observation tunnel-nests for the bees (*i.e.*, boards with grooves routered into one side (8 mm for *O. cornuta*, 7.5 mm for *O. lignaria*, 6 mm for *O. cornifrons*), covered by a layer of clear acetate and, sandwiched with second piece of wood to create a dark tunnel that can be opened to allow for monitoring.

5. Open observation tunnel nest and note completed cells.

6. Temporarily close nest tunnels and apply pesticide at levels of interest to mud.

7. Note new cells created.

8. Open nests and pull out mud partitions divided cells provisioned post-application to measure:
    a. Pesticide residue in pollen-nectar stores (pollen ball), and
    b. Pesticide residue in mud partitions.

9. Remove exposed cells at 15, 20, and 25+ days to assess the movement of the pesticide into bee bread, larval mortality, etc. Depending on the species, full development from egg hatching to adult emergence is completed between 60 and 125 days at 28 to 17 ° C. Higher temperatures will result in faster development, but should not exceed 28 °C.

Using non-*Apis* bees to measure pesticide contamination of pollen and nectar

Using the techniques described above, pollen balls may be removed from the cells of solitary tunnel nesting bees (*e.g. Osmia* spp. or *Megachile rotundata*) placed in shelters deployed in fields or orchards treated with pesticides, including systemic pesticides applied as drench or trunk injection. These managed non-*Apis* solitary bees typically forage in the area immediately surrounding their nest, thereby helping to ensure that the study organism is coming in contact with the treated plants in well-designed field studies. These bees can also be used readily in semi-field studies as they forage readily in enclosures when provided with adequate forage and nesting material (Bohart and Pedersen, 1983; Abel *et al*., 2003).

Female foragers of *Osmia* or *Megachile* spp. may also be netted in front of their nest shelters. If they are returning with pollen, it may be gently scraped or brushed from their abdomens or removed by holding the bee with entomological forceps and applying a vibrating tuning fork to the forceps. Note that unlike honey bees, members of the family Megachilidae, which includes both *Osmia* and *Megachile* genera, carry pollen in long hairs (scopae) on the underside of their abdomens. This pollen is carried dry, unlike honey bees that carry wet pollen with nectar or honey in order to pack it onto their pollen baskets (corbiculae; Vaissière and Vinson, 1994). It is often unknown if wetted pollen may interact with pesticides in the field differently than dry pollen.

In regards to nectar contamination, the crop portion of the alimentary track of non-*Apis* bees can be extracted just as easily as with honey bees. Clearly the amount of nectar that can be recovered will be a bit less in smaller species such as mason bees or leaf-cutter bees, but the procedure is the same as with honey bees. It may be advantageous to anesthetize the foragers prior to squeezing their abdomen gently so as to avoid being stung repeatedly at the same spot though the smaller non-*Apis* species are usually less prone to sting and agile at doing so than honey bees (but this is not true with bumble bee workers).

Field techniques using non-*Apis* bees are presented in greater detail in Chapter **8** on semi-field and field approaches to testing pesticide risk to bees.

Non-*Apis* (solitary species) as an exposure surrogate for *Apis* bees

In certain respects, non-*Apis* bees may serve as a useful surrogate for honey bees in exposure studies. Solitary bees, such as leaf-cutter (*Megachile* spp.) and mason (*Osmia* spp.) bees, typically forage over a much smaller area than honey bees. For example, solitary bees typically forage within a few hundred meters of a nest, rather than two miles as is common with honey bees. Because of this smaller foraging area, it is possible that a field experiment may provide a more accurate picture of potential exposure, even chronic exposure. Where a honey bee colony will forage over potentially 500 hectares or more, if sufficient forage is present, solitary bees will visit flowers as close to the nests as possible and thus be exposed consistently to local field applications and residues.

Summary and Recommendations

Participants of the Workshop agreed that the most significant route of exposure to bees from foliarly applied pesticides is from both dermal contact and oral exposure (of foraging adults, hive adults and larvae) to contaminated pollen, nectar and processed food (*e.g.*, beebread, honey, and larval jelly). For systemic compounds (applied as a seed treatment, soil drench, or trunk injection), the most significant route of exposure is through oral ingestion of residues in pollen, nectar and processed food (*e.g.*, beebread or larval jelly). Other potential routes of exposure include contaminated drinking water and hive material (*e.g.*, contaminated comb wax) and inhalation. For non-*Apis* bee species, unique potential exposure routes include contaminated soil (for solitary ground nesting species and tunnel nesting species that use mud to build cell partitions), contact with sprayed leaves and nesting material that may also be contaminated. Workshop participants agreed that when assessing the major routes of exposure, methods should be conservative enough to account for various potential exposure routes. Unique potential exposure routes, for systemic pesticides, include contaminated fugative dust from seed treatment scenarios, consumption of contaminated aphid honey dew, or possible consumption of contaminated guttation water.

It is important that exposure routes that are formally assessed are in agreement with those that were used to generate the toxicity endpoints available for use in an assessment. Therefore, estimates are needed for contact exposure in adults and dietary exposures for both adults and larvae.

Exposure Estimates

For contact exposure estimates for foliar-applied products, published insect data from direct application exposure studies with honey bees (Koch and Weißer, 1997) can be used to estimate the Predicted Environmental Dose through contact exposure of foraging honey bees (PEDc). Using this data, a worst-case estimate of 1.79 µg/bee is predicted after an application of 1 kg/ha directly to foraging bees.

For non-*Apis* species, Workshop participants recommended using the data for leaf-dwelling and soil-dwelling arthropods from the data developed by Schabacker *et al.* (2005) to address exposure to leaf-dwelling and soil-nesting non-*Apis* bee species, respectively.

For predicting oral exposure to bees for products applied as spray solutions during crop bloom, there is a limited amount of public data available to make an exposure estimate based on predicted concentrations in pollen and nectar. There is however, a larger set of proprietary data that may be available from semi-field studies conducted by pesticide registrants. Therefore, Workshop participants discussed the possibility and value of an industry coalition to compile pollen and nectar residue data from both published and proprietary studies to develop a nomogram that can be used to predict concentrations in pollen and nectar based on field application rates. Preferably, a nomogram such as this would contain both mean and 90^{th} percentile predictions.

Pollen and nectar residue levels, reported as mg/kg can be compared to results from oral exposure toxicity studies with bees if the results of the studies are based on concentrations in diet, *i.e.*, LC_{50}, or as a NOEC (also expressed as mg/kg bee diet). However, if the results from oral exposure toxicity studies are expressed as a median lethal dose (*e.g.*, LD_{50} in μg/bee), then the predicted exposure dose (in μg/bee) can be calculated based on the concentrations in pollen and nectar, and reported (adjusted per) consumption rates from different castes of honey bees.

For systemic compounds applied as seed treatment coating, soil applications or trunk injections, the most significant routes of exposure for adult and larval bees will be through ingestion of pollen, nectar and processed pollen (*i.e.*, beebread or larval jelly) and processed nectar (*i.e.*, honey). Recognizing the limited field data available to develop exposure models, participants of the Workshop considered the proposal by the International Commission for Plant-Bee Relationships (ICP-BR) for a default value of **1 mg/kg in pollen and nectar** (Alix and Lewis, 2010), as a potentially appropriate point estimate of exposure for a screening-level assessment for seed treatment and soil applications. Once again, if the results from oral exposure toxicity studies are expressed as a dose (*e.g.*, μg/bee), then the predicted dose can be calculated based on the concentrations in pollen and nectar coupled with reported consumption rates from different castes of honey bees.

Higher-Tier Studies to Refine Exposure Assessments

When screening level assessment indicates potential risks, higher-tier studies, with applications to bee attractive plant materials are an option to refine exposure estimates for a specific product. A tier 2 [contact] toxicity study of residues on foliage with honey bees may be conducted. In this laboratory study a bee attractive plant (*e.g.*, alfalfa) is sprayed with the formulated product and the bioavailablity and persistence of toxic residues is evaluated at various exposure time-points after application. The results can be used to determine the length of time between application and when bees can be safely exposed to residues on leaves or flowers of a treated crop (i.e., residual toxicity time, referred to as RT).

Refining Oral Exposure of Honey Bees to Foliar-Applied Compounds

Tier 3 semi-field or tunnel tests are recommended to refine the oral exposure assessment for honey bee colonies to both systemic and non-systemic products sprayed on foliage. As discussed in the Hazard –Field section, Workshop participants believed that semi-field studies should use a bee-attractive crop such as *Phacelia*, oilseed rape (*Brassicanapus*), mustard (*Sinapishirta*) or buckwheat (family Polygonaceae). Use of these study/crop scenarios would provide a better opportunity to ensure exposure because the bees would only have the treated crop to forage on for a specified duration. Therefore, the results from a semi-field test would provide data for a realistic, worst-case prediction of exposure of limited duration resulting from labeled use conditions. In these studies, pollen, nectar, bee bread, honey and if desired, larval jelly can be collected and analyzed for residue levels. Unlike honey bee larvae that consume mostly processed pollen and nectar in the form of brood food and/or larval jelly, many non-*Apis* bee larvae consume only raw pollen. As such, in studies using non-*Apis* bees, oral exposure measurements can be obtained directly via the pollen.

Refining Oral Exposure of Honey Bees to Soil Applied and Seed Treatment Systemic Compounds

Once again, a semi-field study is recommended for assessing exposure of honey bee colonies to systemic pesticides delivered via seed dressings or through soil treatments. However, unlike studies with foliar-applied products, for systemic compounds, the actual crop being assessed should be used, (or potential worst case when multiple crops are being considered) since there may be different rates of uptake, distribution and metabolism of a compound in different plant species (i.e., between an attractive surrogate crop such as *Phacelia* and a commercial target crop such as melon). Residue analysis should be timed to coincide with the highest nectar/pollen residues expected in the treated crop based on application timing as well as peak residues during bloom. Residues of systemic pesticides in leaves of trees may be highest several months after soil application, indicating that individual characteristics of the treated crop should be considered in assessing the residues in pollen and nectar. Like semi-field studies conducted with foliar spray products, residues in pollen, nectar, beebread, honey and if desired, larval jelly can be collected and analyzed for residues. The measured residue levels can be used in a refined risk assessment.

Refining Exposure of Non-Apis Bees

If a screening-level risk assessment indicates potential risk, exposure as well as the effect of a compound to non-*Apis* bee species can be refined using field or semi-field study designs. For assessing exposure to pesticides in pollen and nectar, solitary nesting bees such as blue orchard bees (*Osmialignaria*) or alfalfa leafcutter bees (*Megachilerotundata)*, can be used. However, nectar and pollen residue data gained from honey bee trials can also be used to assess exposure for non-*Apis* bees. Similar to studies with honey bees, for foliar-applied pesticides, studies with non-*Apis* bees should be conducted using a bee-attractive crop such as *Phacelia* or sweetclover. Pollen and nectar can be collected directly from the foraging bees. Semi-field or field studies can also be conducted with *Megachile* to evaluate potential [dermal and/or oral] exposure via contaminated nesting material. For assessing exposure to systemic pesticides used as a seed treatment, or applied as a soil treatment or trunk injection, a field study design can be used with the above non-*Apis* species to evaluate worst-case exposure because of the limited foraging range of these species. Potential exposure via soil can also be evaluated using these species.

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Chapter 8 Assessing Effects Through Laboratory Toxicity Testing

Introduction

Toxicity testing in support of a risk assessment process for determining the potential impacts of chemicals to pollinator insects and more specifically bees has typically involved both laboratory and field studies. Initially, tests are conducted that are intended to serve as a screen for whether a chemical represents a potential hazard provided exposure exists. These tests are typically laboratory-based studies and are intended to provide conservative estimates of toxicity based on acute exposures of individual organisms under highly controlled environmental conditions. Based on the likelihood of exposure and the degree of sensitivity of the test species in the initial laboratory tests, more elaborate toxicity tests may be required to understand whether the effects observed in laboratory studies conducted on individual insects extend to the colony/population level. As toxicity testing progresses, the study conditions are intended to be increasingly representative or actual chemical use and reflective of environmentally relevant exposures.

Testing to determine the potential effects of chemicals on non-target organisms has typically relied on the use of surrogate test species since it would not be reasonable or logistically feasible to test all of the species which may ultimately come in contact with the chemical of interest. Selection of a species which is considered a reasonable surrogate has historically been made with consideration of the availability of the species, and consideration of the species' to thrive under laboratory testing conditions. As such, the husbandry/environmental needs of the test species are well known/documented so that tests can be readily conducted and reproduced/replicated. Ideally, the test species should be a relatively sensitive indicator of toxicity; however, it is generally recognized that the test species will not likely be the most sensitive species for which it is intended to represent. Although the European honey bee (*Apis mellifera*) has been used extensively in testing chemicals for potential effects, it is recognized that their biology is considerably different from other non-*Apis* bees (*e.g.*, solitary bees) and other pollinating insects and that these differences may translate into significant differences in how the organism may be exposed/affected. However, the use of suitable surrogate test species facilitates the generation of data that are relatively precise and useful in a regulatory context. The extent to which data from any surrogate test species are considered bias can only be elucidated through equally rigorous studies using other species. Currently, data on other non-Apis bee species is limited and there exists uncertainty regarding the environmental or biological conditions that define potential their relation to pesticides, and ultilmatly their role as a surrogate test species for terrestrial insect pollinators.

To ensure greater consistency in toxicity testing across chemicals, regulatory authorities have established guidelines which outline study design elements that should be considered as well as the nature of data to be collected. This chapter provides an overview of existing toxicity tests and their strengths/weaknesses and then discusses additional studies that could address limitations in the current battery of studies. Although not discussed extensively in this chapter, the intent of toxicity tests is to provide measurement endpoints which can be used to assess the adverse effects from exposure to a particular stressor, *e.g.*, pesticides. Endpoints measured in toxicity tests should provide insight on effects that are likely to impact entire populations/communities rather than effects on a single individual receptor. As such, measurement endpoints drawn from tests should be readily linked to assessment endpoints upon which regulatory authorities base decisions, *i.e.*, impaired survival, growth or reproduction. These assessment endpoints speak directly to maintanience of that taxa at the population/community level. To conserve resources and limit the number of animals required for testing, a tiered process of assessing toxicity has evolved which enables regulatory authorities to focus their resources where they are most needed. Laboratory-based studies are the first tier in evaluating chemicals for their potential effects and depending on the outcome of those studies, more refined studies may be required.

Overview of Laboratory Testing Requirements Among Countries

Overview of Honey Bee Laboratory Testing for Regulatory Purpose in the European Union

To assess the potential hazard of pesticides to honeybees, regulatory agencies in different world regions have developed varied approaches and requirements for hazard testing in support of ecological risk assessment. The requirements for regulatory testing on honeybees for plant protection products (PPP) in the European Union (EU) can be found in Annex II and III of EU Dir 91/414[6]. Additional regulatory guidance is being provided by the EU Terrestrial

[6] [HYPERLINK "http://www.uksup.sk/download/oso/20030409_smernica_rady_91_414_eec.pdf"]

Guidance Document, SANCO/10329/rev 2 final, 2002[7], and in OECD (Please identify the document///effort being referred to) and recently revised EPPO documents[8][9][10]. A new EU Regulation (EC No 1107/2009[11]) regarding PPP registration and replacing the EU Directive 91/414 was published on 21 October 2009, but new data requirements and risk assessment criteria to support the EC No 1107/2009 have not been established.

According to the EU requirements under EU Dir 91/414, testing for pollinators was originally requested to be done in accordance with draft guidance document on honeybee brood tests under semi-field conditions (EPPO Guideline 170 (1992)[12]; however, consistent with SANCO/10329/rev 2 final (2002), honeybee testing could also be conducted through conduct of an acute oral toxicity test guideline (OECD 213, 1998[13]) and acute contact toxicity test guideline (OECD 214, 1998[14]) using young adult honeybees. Where there is only one route of exposure (*e.g.*, oral exposure in case of soil application), testing can be restricted to the relevant route of exposure (SANCO/10329/rev 2 final, 2002). For soil-applied systemic products (*e.g.*, products applied as seed dressing) the acute oral toxicity of the active substance(s) has to be determined as oral exposure is a relevant route of exposure. However, with recent concerns regarding the potential for dust from abraded seed coatings during seeding operations, contact toxicity tests are frequently required as well for products of this formulation. Based on the process followed in the EU, if potential risks to honeybees are identified (*i.e.*, very low LD_{50}) more realistic exposure conditions should be taken into account (*i.e.*, actual exposure concentrations expected in nectar and pollen based on measured residues). If risk estimates exceed regulatory triggers, more refined measures of exposure

[7] [HYPERLINK "http://ec.europa.eu/food/plant/protection/evaluation/guidance/wrkdoc09_en.pdf"]

[8] EPPO, EPPO standards PP1/170-Test methods for evaluating the side effects of plant protection products on honeybees. Bull OEPP/EPPO Bull 31: 323-330 (2011).

[9] OECD. Guidance document on the honey bee (Apis mellifera L.) brood test under semi-field conditions. Series on Testing and Assessment No. 75. ENV/JM/MONO(2007)22 (2007).

[10] EPPO, 2010. Environmental risk assessment scheme for plant protection products, Chapter 10. Risk assessment to honey bees, PP 3/10 (3), OEPP/EPPO, Bulletin OEPP/EPPO Bulletin 40, 1–9.

[11] [HYPERLINK "http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2009:309:0001:0050:EN:PDF"]

[12] [HYPERLINK "http://www.oecd.org/dataoecd/45/44/36036041.pdf"]

[13] OECD/OCDE. 1998. OECD Guidelines for the Testing of Chemicals Honeybees, Acute Oral Toxicity Test. [HYPERLINK "http://www.oecd-ilibrary.org/docserver/download/fulltext/9721301e.pdf?expires=1333215348&id=id&accname=freeContent&checksum=959BEB86B48777CDD914B00E36AA67F0"]

[14] OECD/OCDE. 1998. OECD Guidelines for the Testing of Chemicals Honeybees, Acute Contact Toxicity Test. [HYPERLINK "http://www.oecd-ilibrary.org/docserver/download/fulltext/9721401e.pdf?expires=1333215085&id=id&accname=freeContent&checksum=39EF34D70EBB775A1FAE80D4FA4953EB"]

and effect may be necessary through higher tier studies (*e.g*., cage/tent/tunnel or field studies) with realistic exposure scenarios.

Acute honeybee testing with the formulated product, *i.e*, active ingredient(s) plus inerts, is required if the product contains more than one active substance, or if the toxicity of a new formulation cannot be reliably predicted to be either the same or lower than a formulation tested (EU Dir 91/414, point 10.4.1).

Under EU Dir 91/414, point 8.3.1.2., European authorities may require a bee brood feeding test to assess potential hazard of a subject plant protection product on honeybee larvae. Currently this testing must be carried out when the active substance may act as an insect growth regulator or when effects on the development of immature stages have been identified from other studies in the dossier. Testing of the larvae may be carried out according to the method described by Oomen *et al*. (1992[15]) which is a worst-case screening test under field conditions. If no effects are found the conclusion is justified that no brood damage will occur when using the product. In the case where effects to larvae are determined via the larvae test, further semi-field or field testing will be triggered. (Indeed, OECD guidance document No 75 (OECD, 2007[16]) provides recommendation on the conduct of honeybee brood testing under semi-field conditions.)

Overview of honey bee laboratory testing for Regulatory Purposes in the US

Similar to the EU, the U.S. Environmental Protection Agency (EPA) has developed laboratory-based tests for evaluating the potential toxicity of chemicals to insect pollinators. The U.S. EPA's data requirements for insect pollinator testing are defined in the U. S. Code of Federal Regulations 40 (CFR 40; Protection of the Environment) Part 158 (Data Requirements for Pesticides) Subpart G (Ecological Effects) §158.630[17] and follow a tiered testing approach. Tier 1 consists of an acute contact toxicity test for adult honeybees

[15] Oomen, P.A., A. De Ruijter and J. Van Der Steen. 1992. Method for honeybee brood feeding tests with insect growth-regulating insecticides. Bulletin OEPP/EPPO Bulletin 22: 613 – 616.
[16] OECD. 2007. Series on Testing and Assessment Number 75. Guidance Document on the Honey Bee (*Apis mellifera*) Brood Test Under Semi-field Conditions. ENV/JM/Mono(2007)22
[17] Code of Federal Regulations 40. 2012. Protection of the Environment. Part 158 (Data Requirements for Pesticides. Subpart G (Ecological Effects) § 158.630 (Terrestrial and aquatic nontarget organism data requirements table.
[HYPERLINK "http://ecfr.gpoaccess.gov/cgi/t/text/text-idx?c=ecfr&sid=e2fa3dd8d45333c0c4427f3d556c30f9&tpl=/ecfrbrowse/Title40/40cfr158_main_02.tpl"]

(USEPA Guideline 850.3020[18]). Depending on the outcome of that study and/or whether data on that compound are available in the open literature or on a compound of similar structure, additional studies may be required. Currently, higher tier tests include laboratory-based toxicity of residues on foliage test, *i.e.*, foliar contact toxicity tests (USEPA Guideline 850.3030[19]) and field-based pollinator studies (USEPA Guideline 850.3040[20]). U.S. EPA testing requirements stipulate that the acute contact toxicity tests be conducted using technical grade active ingredient (purity>95%) while higher tier tests are typically conducted using the formulated product.

According to the US CFR40, the acute contact toxicity test with honeybees is required for pesticides with terrestrial, forestry and residential outdoor uses and as indicated previously is conducted using technical grade active ingredient. Worker honeybees of uniform age (1 - 7 days old) serve as the test animals and the guideline is based on methods developed by Atkins *et al*. 1954[21], Atkins *et al*. 1975[22] and Stevenson 1968[23]. Sufficient numbers of bees and treatment levels are used to derive a 48-hour lethal dose to 50% of the organisms tested, *i.e*, LD_{50}. The requirement for a lethality study may be waived if it can be established that the LD_{50} for the subject compound will be greater than 25 micrograms per bee (μg/bee). Limit testing at 25 μg/bee must not result in any mortality; otherwise a definitive LD_{50} should be reported. Test bees are immobilized with CO_2 or N_2 and the test substance is administered as a single topical dose, either via a microapplicator (topical drop) or via whole body exposure to an impregnated dust. A solvent is typically used to administer the test substance and acetone is frequently the solvent selected; it is recommended that the maximum dosage volume not exceed 5 microliters (μL). The bees (minimum of 25 per test level) are closely

[18] USEPA. 1996*a*. Ecological Effects Test Guidelines OPPTS 850.3020. Honey Bee Acute Contact Toxcity. EPA 712-96-147. April 1996. [HYPERLINK "http://www.epa.gov/ocspp/pubs/frs/publications/OPPTS_Harmonized/850_Ecological_Effects_Test_Guidelines/Drafts/850-3020.pdf"]

[19] USEPA. 1996*b*. Ecological Effects Test Guidelines OPPTS 850.3030. Honey Bee Toxicity of Residues on Foliage. EPA 712-C-96-148. April 1996. [HYPERLINK "http://www.epa.gov/ocspp/pubs/frs/publications/OPPTS_Harmonized/850_Ecological_Effects_Test_Guidelines/Drafts/850-3030.pdf"]

[20] USEPA. 1996*c*. Ecological Effects Test Guidelines OPPTS 850.3040 Field Testing for Pollinators. EPA 712-C-96-150. [HYPERLINK "http://www.epa.gov/ocspp/pubs/frs/publications/OPPTS_Harmonized/850_Ecological_Effects_Test_Guidelines/Drafts/850-3040.pdf"]

[21] Atkins, E. L, Jr., L. D. Anderson, and T. O. Tuft. 1954. Equipment and technique used in laboratory evaluation of pesticide dusts in toxicological studies with honey bees. J. Econ. Entomol 47(6): 965-969.

[22] Atkins, E. L., E. A. Greywood, and R. L. Macdonald. 1975. Toxicity of pesticides and other agricultural chemicals to honey bees: laboratory studies. Univ. of California, Division of Agric. Scie., Leaflet 2287: 3800.

[23] Stevenson, J. H 1968. Laboratory studies on the acute contact and oral toxicities of insecticides to honey bees. Ann. Appl. Biol. 61(3): 467-472.

monitored for the first 4 hours and then observed for signs of intoxication for 24 and 48 hrs. EPA recommends testing at least 5 treatment levels and reporting observations on the nature, incidence, time of occurrence, severity, and duration of all observed toxic effects, including death and any other abnormal or unusual signs. Based on the outcome of the acute contact toxicity test, EPA classifies a chemical as to its acute contact toxicity using the following categories based on Atkins *et al.* 1981[24]:

- LD_{50} <2 μg a.i./bee, highly toxic.
- LD_{50} 2 to <11 μg a.i./bee, moderately toxic.
- LD_{50} ≥11 μg a.i./bee, practically non-toxic

If a pesticide formulation contains one or more active ingredients with an acute contact LD_{50} of <11 μg a.i./bee and the use pattern indicates that bees may be exposed, then a toxicity test of residues on foliage (OPPTS Guideline 850.3030[25]) may be required. This guideline is based on the work of Johansen 1977[26] and Lagier *et al.* 1974[27] and is intended to provide data on the residual toxicity of a compound to honeybees. In this study, the test substance is applied to a sample of crop material (alfalfa is preferred) at the typical label rate and at predetermined post-treatment time intervals, the treated foliage is harvested and placed in with caged test bees which are then allowed to forage on the treated plant material during which time bees are monitored and observed for mortality and signs of intoxication. Mortality is determined after 24 hours of exposure to the treated foliage. The treated foliage is then typically minced and mixed, then divided into 15-gram portions which is placed into cages containing at least 25 forage (1 - 7 days old) bees. The guideline recommends that each treatment consists of at least six replicates. Foliage is collected at 3, 8 and 24 hrs after application. If the mortality of bees exposed to 24-hour old residues is greater than 25%, sampling should continue at 24-hr intervals until mortality of bees exposed to treated foliage is not significantly greater than controls. During the observation period, all signs of intoxication, other abnormal behavior, and mortality should be recorded and reported by treatment and by time of occurrence. During the study period, bees are provided 50% sucrose/water *ad libitum*.

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[24] Atkins, E. L., D. Kellum and K. W. Atkins. 1981. Reducing Pesticide Hazards to Honey Bees: Mortality Prediction Techniques and Integrated Management Strategies. University of California Division of Agricultural Sciences. Leaflet 2883.
[25] *Ibid* USEPA. 1996*b*
[26] Johansen, C. *et al.* 1977. Bee Research Investigations. Dept. of Entomology, Washington State University, unpublished, 22 pp.
[27] Lagier, R.F. *et al.* 1974. Adjuvants Decrease Insecticide Hazard to Honey Bees. College of Agriculture Research Center, Washington State University Bulletin 801, 7 pp.

Beyond the toxicity test of residues on foliage, if any of the following conditions are met, EPA may require a pollinator field study (OPPTS Guideline 850.3040[28]):

- Data from other sources (experimental testing programs, university research, registrant submittals, *etc.*) indicate potential adverse effects on colonies, especially effects other than acute mortality (reproductive, behavioral, *etc.*);
- Data from residual toxicity studies indicate extended residual toxicity.
- Data derived from studies with terrestrial arthropods other than bees indicate potential chronic, reproductive or behavioral effects.

Field pollinator testing is intended to examine the potential effects of a chemical on the whole honey bee colony, and the nature of these studies is discussed in the field hazard chapter (Chapter X).

Uncertainties in Current Testing Paradigms

At this time, the current EPA hazard testing scheme does not include specific guideline studies for assessing the direct oral toxicity of pesticides to cover potential exposure through consumption of nectar as the EU scheme does. Additionally, acute toxicity testing of honeybees in either the U.S. or the EU has not formally included studies examining the potential effects of pesticides on honeybee larvae (brood). Additionally, the current laboratory test guidelines in the U.S. focus on contact toxicity and do little to provide information on the toxicity of compounds that may be ingested through consumption of pollen/nectar. However, the contact toxicity test may result in some ingestion of test material through normal grooming behavior. While test guidelines stipulate that sublethal effects must be reported, the typical endpoint reported from the acute toxicity tests is the LD_{50} and rarely is a median effect concentration (EC_{50}) based on sublethal effects reported. Given that the current U.S. test guidelines are designed to yield regression-based endpoints, *i.e.*, LD_x values, endpoints such as no-observed-adverse-effect concentrations (NOAEC) and lowest-observed-effect concentrations (LOAEC) which require hypothesis testing are not likely since treatments are sufficiently not replicated.

Also, as noted earlier, under the U.S. testing process, the honeybee is used as a surrogate for other pollinator insects and for terrestrial invertebrates. In the EU however, specific test

[28] *Ibid* USEPA. 1996*c*.

guidelines are available for examining the effects of pesticides on non-target arthropods independent of the studies examining toxicity to honeybees. Uncertainties regarding the use of honeybees as surrogates for other non-*Apis* bees were identified at the Pellston workshop. These uncertainties centered on the fact that the life history and social biology of honey bees is significantly different from that of other bees and arthropods. At this time, it is uncertain whether honeybees, and the data generated on them are reasonable surrogates for other non-*Apis* bees or insect pollinators in general (*i.e.*, whether laboratory studies conducted with *A. mellifera* provide endpoints which are sufficiently protective of the range of sensitivities that may exist among non-*Apis* bees or other insect pollinator insects and/or terrestrial invertebrates in general). However, it was noted by Pellston participants, that since laboratory studies are intended to examine the intrinsic toxicity of a chemical to a particular test organism, differences in the biology of the test organism relative to those species for which it is intended to serve as a surrogate may not be critical. **Table 1** provides a comparison of the acute laboratory toxicity tests (OECD 213, OECD 214 and OPPTS 850.3020) currently required by regulatory authorities in the EU and U.S.

Table X. Comparison of acute contract test guidelines (OECD 214 and EPA OPPTS 850.3020) and acute oral test guideline (OECD 213)

	OECD 214 (acute contact)	EPA OPPTS 850.3020 (acute contact)	OECD 213 (acute oral
Status and background	Adopted 21 September 1998 Based on EPPO GL 170 (1992) and improvements considered made by ICPBR (1993) Other GLs considered: SETAC (1995), Stute (BBA) (1991), EPA OPPTS 850.3020 (1995).	Public draft April 1996 Based on OPP 141-1 (1982)	Adopted 21 September 1998 Based on EPPO GL 170 (1992) and improvements considered made by ICPBR (1993) Other GLs considered: SETAC (1995), Stute (BBA) (1991), EPA OPPTS 850.3020 (1995).
Test species and test organisms	Young, healthy, adult worker bees (*Apis mellifera*), same race, similar age and feeding stage, from queen-right colony, known history. Bees collected from frames without brood are suitable. Bees should not have been treated chemically for at least 4 weeks.	Young test bees, 1-7 days old (*Apis mellifera*), may be obtained directly from hives or from frames kept in an incubator, from same source	Young, healthy, adult worker bees (*Apis mellifera*), same race, similar age and feeding stage, from queen-right colony, known history. Bees collected from frames without brood are suitable. Bees should not have been treated chemically for at least 4 weeks.
Test cages	Clean and well-ventilated made of any appropriate material, *e.g.*, stainless steel, wire mesh, plastic, disposable wooden cages. Groups of 10 bees	Test chambers may be constructed of metal, plastic, wire mesh, or cardboard, or a combination of these materials. Groups of at least 25 bees	Clean and well-ventilated made of any appropriate material, *e.g.*, stainless steel, wire mesh, plastic, disposable wooden cages. Groups of 10 bees
Handling, feeding, preparation	Food - *ad libitum* – as sucrose solution (50% w/v), *e.g.*, via glass feeders Bees may be anaesthetized with carbon dioxide	A 50% sugar/water solution should be provided *ad libitum* (purified or distilled water should be used). Bees may be anaesthetized with carbon dioxide	Food - *ad libitum* – as sucrose solution (50% w/v), *e.g.*, via glass feeders Feeding system should allow recording of food intake (*e.g.*, glass tubes 50 mm long, 10 mm

	(CO_2) or nitrogen (N_2) for application. Amount should be minimal Moribund bees should be rejected before testing	(CO_2) or nitrogen (N_2) for application.	wide, and narrow end) Bees may be starved for up to 2h before test initiation Moribund bees should be rejected before testing
Solvents	Test substance applied as solution in a carrier, *i.e.*, organic solvent – acetone preferred – or a water solution with a (commercial) wetting agent. Two separate control groups, *i.e.*, water and solvent /dispersant	A solvent is generally used to administer the test substance. The solvent of choice is acetone (or other volatile organic solvents) Two concurrent control groups, *i.e.*, water and solvent (or carrier) control	Test substance applied as 50% sucrose solution in a carrier ie organic solvent (*e.g.*, acetone), emulsifiers or dispersants at low concentration up to max 1% should not be exceeded. Two separate control groups, *i.e.*, water and solvent /dispersant
Test and control groups	Normally 5 doses in geo-metric series with a factor ≤ 2.2 covering the range of LD_{50} for definitive test (ranger-finder proposed) Minimum of 3 replicates with 10 bees for each dose rate and control (Minimum of 30 bees for each dose) Max. ≤ 10% control mortality at test end	A minimum of 5 dosage levels spaced geometrically. Recommended spacing for each dosage level to be at least 60 percent of the next higher level. Three or more dosages should result between 0 to 100% mortality. Minimum of 25 bees for each dosage. Max. ≤ 20% control during the test	Normally 5 doses in geo-metric series with a factor ≤2.2 covering the range of LD_{50} for definitive test (ranger-finder proposed) Minimum of 3 replicates with 10 bees for each dose rate and control (Minimum of 30 bees for each dose) Max. ≤ 10% control mortality at test end
Limit test	100 µg ai/bee in order to demonstrate that the LD50 is greater than this value.	25 µg ai/bee in order to demonstrate that the LD_{50} is greater than this value.	100 µg ai/bee in order to demonstrate that the LD_{50} is greater than this value.
Toxic	At least 3 dose rates with 3 x 10 bees to	A concurrent positive control is not required.	At least 3 dose rates with 3 x 10 bees to

standard	demonstrated,*e.g.*, the toxic standard, dimethoate, is within the reported contact LD_{50} of 0.10-0.30 µg ai/bee (Gough *et al*. 1994). Other toxic standards are acceptable.	A lab standard is recommended; also when there is a significant change in source of bees.	demonstrated eg the toxic standard, dimethoate, is within the reported contact LD_{50} of 0.10-0.35 µg ai/bee (Gough *et al*. 1994). Other toxic standards are acceptable.
Exposure	1 µL per bee applied on dorsal side of thorax (higher volumes, if justified) via micro-applicator. Temperature: 25±2°C Relative humidity: 50-70% Test duration: 48h. (If mortality increases by > 10% between 24h and 48h the duration is prolonged to maximally 96h provided that the control does not exceeding 10%.)	5 µL per bee should not exceeded Temperature: 25-35°C Relative humidity: 50-80% Test duration: 48h	100-200 µL per 10 bees of 50% sucrose solution in water (or higher) provided for 3-4 (max. 6)h. Amount consumed amount is measured. Temperature: 25±2°C Relative humidity: 50-70% Test duration: 48h. (If mortality increases by > 10% between 24h and 48h the duration is prolonged to maximally 96h provided that the control does not exceeding 10%.)
Observations	Mortality at 4h, 24h, 48h, and potentially at 72h and 96h. Abnormal behavioural effects during the test period should be recorded.	Mortality at 4h, 24h, 48h All signs of intoxication and other abnormal behaviour (*e.g.*, ataxia, lethargy, hypersensitivity) during the test period should be recorded.	Mortality at 4h, 24h, 48h, and potentially at 72h and 96h. Amount of diet consumed per group should be measured to determine palatability of diet. Abnormal behavioural effects during the test period should be recorded.
Data	Range-finding data	Range-finding data	Range-finding data

reporting	LD$_{50}$ plus 95% confidence limits, *i.e.*, at 24h, 48h and, if relevant 72h and 96h (in µg test substance per bee) and slope of curves Mortality statistics (*e.g.*, probit analysis, moving-average, binominal probability) Other biological effects and any abnormal bee responses Deviations from test guideline	LD$_{50}$ plus 95% confidence limits, *i.e.*, at 24h, 48h and, and slope of curves, goodness-of-fit test results Mortality statistics (*e.g.*, probit analysis, moving-average, binominal probability) Signs of intoxication and other abnormal behaviour. Deviations from test guideline	LD$_{50}$ plus 95% confidence limits, *i.e.*, at 24h, 48h and, if relevant 72h and 96h (in µg test substance per bee) and slope of curves Mortality statistics (*e.g.*, probit analysis, moving-average, binominal probability) Other biological effects and any abnormal bee responses Deviations from test guideline

At this time, a variety of laboratory-based study methodologies are available to examine the acute oral and contact toxicity of chemicals to honey bees. As noted previously, it is important that studies conducted for regulatory purposes provide a consistent and clear way to assess the toxicity of chemicals without confounding effects from the study conditions themselves. The honey bee has been used as a surrogate for testing of potential effects because bees are readily available, their husbandry needs are well known, and the bee performs well under confined laboratory conditions. Additionally, considerable testing has been conducted with the honey bee under relatively standardized conditions which has resulted in a sizeable database on the toxicity of a wide range of chemicals. This standardized toxicity data enables risk assessor to compare the relative toxicity of chemicals to bees across chemical classes with highly divergent modes of action. Work shop participants believed that since tier 1 laboratory studies (*i.e.*, acute lethality studies) often serve as a screen for determining whether chemicals represent a potential hazard to bees, there is a critical need to ensure that study designs are harmonized across testing guidelines; and, that these tests are designed to provide the highest quality data with the least amount of variability.

Limitations and suggested improvements for Tier 1 testing

Adult *Apis mellifera* Worker Acute Toxicity

Exposure of honeybees can be dermal from from direct overspray while the bees are foraging, by contact with contaminated surfaces of the plant, or by intake of contaminated pollen and nectar. The hazard posed by short-term exposures can be assessed using acute toxicity tests in which the LD_{50} is calculated. As discussed in the preceding section, acute honeybee testing under laboratory conditions has been conducted for many decades according to many different test guidelines and published methods, *e.g.*, OECD, EPPO 170 (1992, and updated in 2010), SETAC (1995), Stute (1991), EPA OPPTS 850.3020 (1996). Workshop participants considered the OECD test guidelines (OECD 1998) the most robust of those available for assessing the acute toxicity of pesticides to honeybees.

Acute honeybee tests performed according to OECD 213 and OECD 214 can be designed as limit tests or as dose-response studies (with a minimum of 5 doses and a minimum of 3 replicates of 10 bees at each dose). The bees are held under controlled temperature and humidity conditions and mortality and behavior is monitored for a minimum of 48 hours (this is extended if effects are prolonged). The reported data includes the LD_{50} (with 95% confidence limits), at 24h, 48h and, if relevant 72h and 96h time points (in µg test substance per bee), the slope of dose-response curves, and any other observed abnormal bee responses. Both tests include both a control (treated with the same concentration of solvent as in the treated doses) and a toxic standard (*e.g.*, dimethoate) with defined acceptance criteria.

The OECD acute contact test (214) involves direct application of the test substance (active ingredient or formulation), usually as a 1 µl drop, diluted in an organic solvent or water as required, applied directly to the dorsal thorax of the bee. Among the advantages of the OECD 214 acute contact test guideline are:

- replication (at least 3 replicates);
- no in-hive treatments for 4 weeks prior to use in a study are permitted;
- higher number of test organisms is specified (30 bees);
- prescriptive environmental conditions;
- stringent control mortality is specified (10%);
- a toxic standard is required and validity criteria are stated; and,
- test duration is prolonged in case of delayed effects.

The only internationally accepted oral acute toxicity test guideline is OECD 213. The test is similar in design to the OECD 214, acute contact toxicity test, but consists of group feeding of a known volume of treated sucrose solution over a maximum period of 6 hours to the replicate bees within a cage and then untreated sucrose is supplied *ad libitum*. Group feeding can be used to administer the dose of test substance because honeybees exhibit trophollaxis, *i.e.*, the transfer of food among members; the applicability and repeatability of this is demonstrated by the toxic reference which is stable within a testing facility. The test requires monitoring of the actual intake of the treatment to determine the intake of the test substance per bee as some pesticides, such as pyrethroids are repellent and the total dose may not be consumed.

Possible improvements

Participants of the Workshop discussed the limited number of cases which would compel specific deviations from the OECD guideline for successful handling in the laboratory conditions, such as with the Africanized bee present in South America for example (Nocelli personal communication). However changes in study design can affect outcomes and reliability of the resulting data. Before data generated from modified study designs can reliably be used in risk assessment, methodology and the resulting data should undergo a separate validation exercise (*e.g.*, determination of appropriate toxic reference and control data).

Adult Oral Chronic toxicity

Undertaking an adult oral chronic toxicity study is an optional refinement step in the proposed risk assessment scheme. Currently there is no standardized guideline for this test, but method proposals and study design elements from acute toxicity tests may be found in a number of publications, *e.g.*, Schmuck 2004, Suchail *et al.* 2001[29], Moncharmont *et al.* 2003, Alioune *et al.* 2009 and the EPA Guideline OPPTS 850.3020. However before undertaking such studies, care should be paid to a number of factors:

[29] Suchail S, Guez D, Belzunces LP (2001) Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites in *Apis mellifera* . Environmental Toxicology and Chemistry; 20:2482-2486.

- There is no standardized duration for the study considering that the longevity of bees differs between summer and winter. It is currently recommended that the study be performed over a10- to 14-day duration to ensure high control survival.
- To achieve a 10- to 14-day study duration, a mixed pollen (protein source) and sucrose (carbohydrate source) diet may be required.
- Some pesticides may induce reduced food intake due to repellency (*e.g.*, pyrethroids) and the longevity of the bees may be affected by the reduced food intake due to repellency rather than reflecting a toxic effect of the pesticide. Therefore, food intake has to be assessed in parallel with mortality on a daily basis. The pattern of exposure may affect the observed toxicity (*e.g.*, a *sin*gle dose per day verses continuous exposure). Continuous exposure could mean: 1) dosed diet *ad libitum* or, 2) a fixed amount of dosed diet daily (*e.g.*, 2 hours plus untreated diet during the rest of the time). It is recommended that both approaches (single dose and continuous exposure) are used until sufficient data have been generated to clarify which is the most reliable.

Proposal for a chronic adult oral toxicity study

Below are elements of a chronic oral toxicity test proposed by Workshop participants:

- Newly emerged bees up to 2-days old should be used (these can be emerged from the brood comb in an incubator).
- Cages should be well ventilated and sufficiently large to allow the bees to move around freely.
- Minimally, three replicates per dose and 10 bees per cage should be used; however, it is important to note that statistical power is based on the number of replicates (treatment units) and not the number of bees within the treatment unit.
- There should be a minimum of 5 dose rates (treatment levels) to achieve a dose-response curve for the test item and to allow generation of the lethal concentration to 50% of the bees tested, *i.e.*, LC_{50}, a no-observed-effect-concentration

(NOEC), and sufficient doses to verify the LC_{50} of a toxic reference chemical (*e.g.*, dimethoate).

- The test substance should be dissolved in the aqueous sucrose solution (using a maximum of 1% solvent (*e.g.*, acetone) if required.
- If a solvent is required to dissolve the test substance, then a suitable solvent control should be run in addition to a negative control concurrent with the treatments Therefore, both an untreated sucrose (50% w/v) control and, if a solvent has been used to suspend the test item in sucrose, a sucrose-solvent control containing the same maximum concentration of solvent as the test item should be used.
- A protein supplement may be used in the 50% w/v sucrose if this ensures control mortality is acceptable at 10 days.
- As a chronic toxicity test, concentrations/levels should be selected to minimize mortality and facilitate measurement of sublethal effects. While the test may provide information on the LC_{50}, a median effect concentration (EC_{50}) based on sublethal effects (*e.g.*, impaired behaviour, growth) should be a primary focus of the study.
- Two dosing methods should be considered:

  1. The volume of treated sucrose should be sufficient to allow *ad libitum* feeding for a 24 hr period (continuous dosing).
  2. A small volume of treated sucrose (*e.g.*, 20µL/bee) should be offered for 2-4 hours each day and then replaced with untreated sucrose (daily dosing). It may be necessary however, to starve (fast) the bees before providing the treated sucrose solution to ensure that the dosed test solution will be completely consumed by the test organisms).

- The amount of treated sucrose offered to the bees and the amount remaining each day should be recorded. The dose consumed should be determined by comparing the weight of the dose remaining in the glass feeders with the weight of a known volume of the test solutions. The composition of the feeders is an important consideration since, depending on the test chemical, material other than glass can interfere with the availability of the test substance.

- During the test period, the bees are kept in the dark (except during observations) in an incubator at 25±2°C and 60-80% relative humidity.
- Mortality and sublethal effects should be assessed at 24-hour intervals after the start of the test for up to 10 days. Sublethal effects should be assessed according to appropriate categories. Control mortality should be not greater than 15%.
- As with any toxicity test protocol, the stability of the test material must be considered when determining the exact methods used in the study. Ideally, nominal concentrations/levels of the test chemical should be verified through analytical measurements.
- The source of the test bees must be recorded, and to the extent possible, disease/parasite loads should be minimized. Any treatments (*e.g.*, antibiotics) other than the chemical of interest must be documented and must be consistent across treatments/controls. To the extent possible, the bees should be from a single colony and/or derived from colonies with sister queens. As with all studies, bees should be assigned to treatment groups randomly.

Method for testing pesticide toxicity to honeybee brood in laboratory condition

The *in vitro* honeybee brood test provides quantitative oral/contact toxicity data on larvae for active ingredients or formulated products. These data should be used in an appropriate brood risk assessment scheme. *In vitro* larvae tests have been developed by Rembold and Lackner (1981) and used for the assessment of pesticides by Wittmann (1982). Aupinel *et al*. 2005 improved this method in several aspects. Below are elements of an *in vitro* honey bee brood test based upon Aupinel, *et al*. (2005) with suggested modifications from the Workshop participants.

- Larvae at the L1 (first instar) stage are fed standardized amounts of a semi-artificial diet. Test items (pesticides or other products of interest) are incorporated into the food at different concentrations within an appropriate range in order to compute the following end points for larvae (L1 to L5), pupae (L5 to adult emergence) and adults (emergence to day 22 post-emergence): LC_{50}, LD_{50} and NOEC (the NOEC will be the principle target endpoint).

- The reference product is typically dimethoate.

Larvae termination and collection

- For one replicate, larvae are collected from a unique colony. Test colonies have to be healthy and must not show any visible clinical symptoms of pests, pathogens, and/or toxin stress. Tests should be conducted with summer larvae during a period from the middle of spring to the middle of autumn (the exact time of year varies by location). No varroa treatment with the exception brood removal should be applied within the 8 weeks preceding the beginning of experiments.
- At Day -3 (prior grafting, Fig. 4), the queen of the chosen colony is confined in its own colony onto a comb. This can be done using an excluder cage into which a comb (dark preferred) containing empty cells is placed or by using a smaller push-in cage (~10 × 10 cm) which can be used to confine a queen on a given section of comb containing empty cells. In both cases, the comb is placed close to other combs containing brood (Fig. 1).
- At Day -2, with the verification that there are eggs, the queen is removed from the cage 22-26 hours after she was encaged. To ensure that larvae are available at Day 1 of the study it is recommended to cage the queens of 2 or 3 colonies in the event a queen is laying few or no eggs. Based on queen vigour, the queen's isolation time can be reduced in order to minimize variability in larval size (age).
- The comb containing the eggs is left caged to prohibit the queen from ovipositing further on the comb on the same position near the brood frames. The eggs develop until the hatching larvae at Day 1.

- At Day 1 (Fig. 3), the comb containing first instar larvae is transferred from the hive to the laboratory for grafting. As L1 larvae are subject to dessication a wetted towel should be placed around the comb.

Preparation of rearing material

Rearing Cells

- Larvae (≤1 day old) are reared in polystyrene grafting cups (common among beekeeping equipment supply companies. Cells with rounded bottoms are best) having an internal diameter of approximately 9 mm. Before use, the cells are washed and sterilized in 0.4% MBC (methyl benzethonium chloride) water solution, or ethanol and rinsed in sterile water then dried in a laminar-flow hood. Each larva is placed into a well of a 48-well tissue culture plate.
- Larvae plates with lids closed, are placed into a larval chamber such as a hermetic chamber (*e.g*., Plexiglas desiccator, a plastic container, *etc*.) into which a dish having a potassium sulphate (K_2SO_4) saturated solution is placed to maintain a water saturated atmosphere (>90% relative humidity). The larval chamber is placed into an incubator at 34,5°C. It is important that this temperature is maintained within a small range since temperature can affect the toxicity of pesticides to immature bees (Medrzycki *et al*. 2010).

Larval Food

- The food is composed of three diets for different days of the study with Diet A following the recipe of Vandenberg and Shimanuki (1987) and subsequent diets modified from this basic diet.
    - Diet A (Day 1): 50% fresh royal jelly + 50% aqueous solution containing 2% yeast extract, 12% glucose and 12% fructose. A recipe for 20 g diet contains 10 g royal jelly, 1.2 g glucose, 1.2 g fructose, and 0.2 g yeast extract mixed in 7 mL H_20.
    - Diet B (Day 3): 50% fresh royal jelly + 50% aqueous solution containing 3% yeast extract, 15% glucos and 15% fructose. A recipe for 20 g diet contains 10 g

royal jelly, 1.5 g glucose, 1.5 g fructose, and 0.3 g yeast extract mixed in 7 mL H_20.

- Diet C (from Days 4 to 6): 50% fresh royal jelly + 50% aqueous solution containing 4% yeast extract, 18% glucose and 18% fructose. A recipe for 21 g diet contains 10 g royal jelly, 1.8 g glucose, 1.8 g fructose, and 0.4 g yeast extract mixed in 7 mL H_20.

General Information Regarding Diet Preparation

Royal jelly can be stored frozen at -20°C in small aliquots to avoid multiple freezing which causes a change in the sugar crystals. It should be thawed by placing it at 4°C overnight, or at room temperature for 1-2 hrs. Reverse osmosis water or distilled water should be used, boiled for 10 min, and cooled to 45-55 °C (cool enough for hands to touch) prior to using it for mixing. Water, sugars and yeast should be mixed thoroughly (all solid materials should be broken up with a sterile spatula) in lab ware (preferably glass lab ware such as a beaker) that has been autoclaved. The mixture should be vortexed for 30 seconds. Once the bubbles have settled, the total volume should be adjusted to 10 mL with the prepared water. Finally when the mixture has room temperature, 10 g of royal jelly should be added to the mixture and the mixture vortexed for 30 seconds. The diets prepared for a test should be stored in a refrigerator at ~5-10°C during the test.

Pupation and emergence

- At Day 7 (prepupal stage), the plates with open lids are transferred into a pupal chamber (*i.e.*, a hermetic Plexiglas desiccator, a plastic container, *etc.*). The chamber should be maintained with a saturated atmosphere (~75% relative humidity) this can be achieved by placing a dish with a NaCl saturated solution into the chamber.
- The container is then placed into an incubator at 34,5°C.

- At Day 15, each plate is transferred into an emergence box (~11 × 15 × 12cm) with a cover that is aerated with wire gauze. The emergence chamber should contain a piece of comb (~3 × 5 cm) which attracts the emerging bees. Emerging bees are fed *ad libitum* with a sucrose syrup solution (50% sucrose/distilled water by volume) that is supplied in an 2ml eppendorf tube with a hole below. The emergence box is returned to the pupal chamber.

Grafting and feeding of larvae

- The rearing cells in the well plate are prepared by pipetting 20 µl of Diet A into each cell. The comb is placed angular on a clean table and a cold light or LED light is used for illumination to prevent larvae from drying.
- The grafting of the L1 larvae is performed by quick transfer from the comb to each plastic cell cup and placed on the surface of the diet using a grafting instrument of choice (a grafring spoon, paint brush size 00, Chinese grafting tool, *etc*.).
- If grafting is performed from several combs or a comb is not use for a moment it should be covered by the wetted towel. The grafting should be performed randomly to maintain treatment heterogeneity.
- When a plate is completed with 48 larvae, it is placed into the larval chamber and then into the incubator immediately.
- The larvae are fed once a day (except at Day 2) at the same time of day (+/- 1 hour) 3 different diets in different amounts using a stepwise pipette with sterile tips (see Fig. 4 for feeding timeline) following the scheme given in Figure 4. Prior to administration to the larvae, the diet is warmed to 34,5°C by placing in the incubator 1 hour prior feeding. The diet should be pipetted on the inner side wall of the cell to slide slowly down in order to avoid the larvae from drowning. It must be avoided that diet is placed on the larvae to prevent the blocking of the spiracles.

Experimental Groups

- The experimental unit is a single larvae in a cell and a treatment group consists of minimum 24 larvae (half of a 48 tissue culture plate). For each test, the following treatment groups should be used:
  - 1 control diet without solvent (24 larvae)
  - 1 control diet with solvent (24 larvae).
  - 5 test item concentrations (24 larvae each)
  - 1 reference treatment with dimethoate (24 larvae)

Each test (all 8 groups of test larvae) should be replicated across 3 independent colonies (unrelated queens).

Preparation of the pesticide solutions

- The test pesticide is dissolved in water (the preferred solvent) or acetone if the pesticide is not water soluble. If a solvent other than water is used, a second solvent control group must be used consisting of control larvae fed with diet containing the solvent at the same concentration as the treated samples.
- Dilutions of the stock solutions are made with non-chlorinate, sterile drinking water using disposable pipette tips equipped with a filter. The amount of test solution administered must not exceed 10% of the final volume. In all cases, one must include the same final volume of water or solvent in all treatments and controls.

Treatments

- In acute toxicity tests, larvae are treated at Day 4 with Diet C containing the test item solutions at their respective test concentrations.

- For chronic toxicity tests, larvae are treated daily (except Day 2) with the diets containing the test item solutions at test concentrations. In order to assess the adequate endpoints (NOEC and LC_{50}), it is recommended to run a preliminary experiment where the appropriate concentrations of the test preparation, vary geometrically at 5 to 10 different concentrations, can be determined.

Toxic Reference

- The toxic reference is typically the organophosphate dimethoate:
  - in acute toxicity tests: 3 µg/larva is mixed with Diet C and provided at Day 4,
  - in chronic toxicity tests: it is mixed with the three diets at test concentrations of 20 µg / kg diet.

Definition of Mortality

- LARVA: An immobile larva (not breathing or moving when viewed under a dissecting scope) is recorded as dead. If a larva's mortality is in doubt, examine the larva the following day.
- PUPA: A non-emerged individual at Day 22 is considered as dead during the pupal stage.
- ADULT: An immobile adult which does not react to a tactile stimulation is recorded as dead.

Mortality Assessments

LARVA: Daily (except Day 2) when larvae are fed, all dead larvae are removed for sanitary reasons. Specific mortality checks are made according to the type of test (acute or chronic). In the acute test where exposure is at Day 4, a first mortality check is made at Day 4 in order to replace the dead larvae before they have started consuming the diet containing the insecticide. Mortality must also be recorded at Days 5, 6 and 7. In the test with chronic exposure mortality is noted at Day 7.

PUPA: Non-emerged bees are counted at Day 22.

ADULT: short-term survival: living [emerged] adult bees and dead adults which left their cell and show a normal development are counted at Day 22.

Long-term survival: living adult bees and dead adults are assessed daily through 10 days post-emergence. Typically, control mortality increases from day 12 to 14.

Validity range of data

- For the test to be considered valid, bees fed the control diet must adhere to the following:
  - Larvae - ≤10% mortality (number of dead larvae/24)
  - Pupae - ≤20% mortality (number of dead pupae at Day 22/24)
  - Adult- ≤10% mortality (number of dead adults at Day 10 post-emergence/total number of emerged adults)

If the mortality in the control groups is higher than that outlined above, the test is invalidated.

The rate mortalities within the dimethoate control should be:

- Acute test: ≥50% mortality at Day 6 for larvae exposed to 3 µg dimethoate / larva at D4
- Chronic test: ≥50% cumulative mortality at Day 7 after exposure to 20 mg dimethoate/kg diet.

The calculated LC_{50} must be in each case between the concentrations tested; the LC_{50} must not be extrapolated outside of the tested concentration.

LD_{50} and LC_{50} Calculation

- Mortalities are expressed in percentage of the reference populations after an adjustment according to the Abbott formula (1925):

$$M = \frac{(P - T)}{S} \times 100 \text{ EQ1: raw mortalities}$$

$$M = \frac{(\%P - \%T)}{100 - \%T} \times 100 \text{ EQ2: percent mortalities}$$

- M is the adjusted mortality expressed in percent of the initial population, initial number of larvae (24) for a larval mortality, number of living pre pupae at Day 7 for pupal mortality, number of emerged [adult] bees at Day 22 for an adult mortality
- P: mortality due to the treatment
- T: control mortality
- S: surviving number in control
- %P: mortality percentage due to the treatment
- %T: control mortality percentage

The results will be analysed using regression and/or probit modelling. All raw and adjusted data must appear in the study report. The lethality graphs and their equations must be reported. The results should include LC_{50} values for 24 and 48h expressed in terms of µg per individual (for the acute test), and for a LC_{50} in µg per litre of solution (ppb) for the chronic test. These calculated variables should include their respective 95% confidence intervals.

Determination of the NOEC

The NOEC is the highest concentration which does not induce mortality significantly higher than that observed in controls. This analysis is typically performed using a Chi2 test (1 tail test, at an alpha of 0.05).

3. Defined and defended endpoints needs further clarification

- Larvae (from grafting until defecation): NOEC/LC_{50} and weight at defecation
- Pupae (from defecation to adult emergence): NOEC/ LC_{50} and weight at emergence
- Adult (from emergence until mortality): NOEC/LC_{50} (Should we do mortality or just include an endpoint? Say, day 18 or 22?)

Adult Toxicity Testing with non-*Apis* Bees

As discussed previously, there is always uncertainty regarding the extent to which a surrogate test species, such as the honeybee, is a sensitive indicator of the many other species it represents. Data currently available suggest that adult non-*Apis* bees are similar in pesticide sensitivity to *A. mellifera* when bodyweight is taken into account. Caution must be added as the dataset to date is weighted to pesticides of older chemistries. **Figure 11** shows the relative toxicity (contact LD_{50}) of 21 pesticides to bumble bees and solitary bees in comparison to the honeybee based on weight. **Figure 12** depicts the decline in toxicity of residues on foliage for honeybee adults compared to the solitary alfalfa leaf-cutter bee (*Megachile rotundata*) and the alkali bee (*Nomia melanderi*). **Figure 13** depicts the median lethal doses of sprayed residues of four pesticides (clothianidin, imidacloprid, lambda cyhalothrin and spinosad) to *A. mellifera*, *M. rotundata*, and *O. lignaria*. These data suggest that the toxicity of these pesticides falls within and order of magnitude of the values for *A. mellifera*. However, consideration may be given to testing non-Apis bees when there is evidence to suggest that the honeybee is not likely to be a reasonable surrogate. When selecting species to be used in the laboratory it is important to consider their availability, ease of handing and survival under controlled laboratory conditions. Therefore, it is recommended that relevant and sensitive species used are those that are either reared for commercial use or can be readily cultured under laboratory conditions.

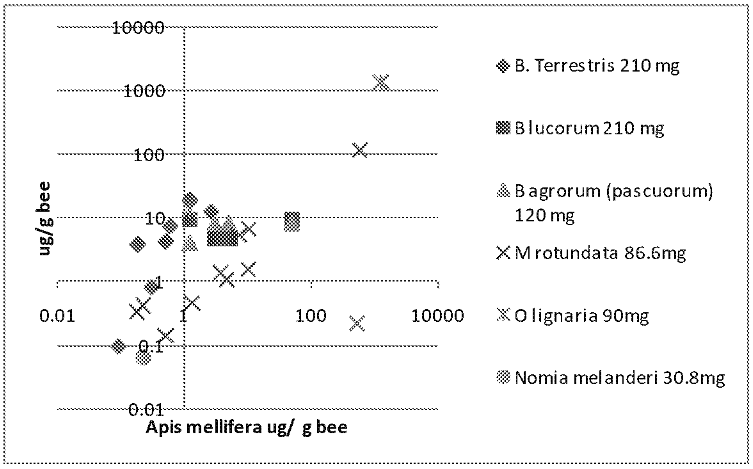

Figure 11. Comparison of the contact toxicity (LD_{50}) of 21 pesticides to adults of *Apis mellifera* , 3 species of the social bee Bombus and 3 species of solitary bees (Osmia, Megachilidae and Nomia).

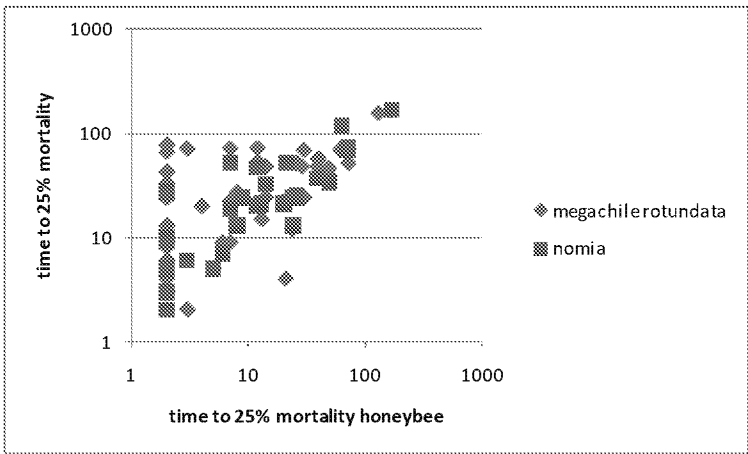

Figure 12. Comparison of the toxicity of pesticides to adults of *Apis mellifera* with the solitary bees *Megachile rotundata* and *Nomia melanderi* based on time for sprayed residues to decline to a concentration causing 25% or less mortality (Johansen *et al.* 1986).

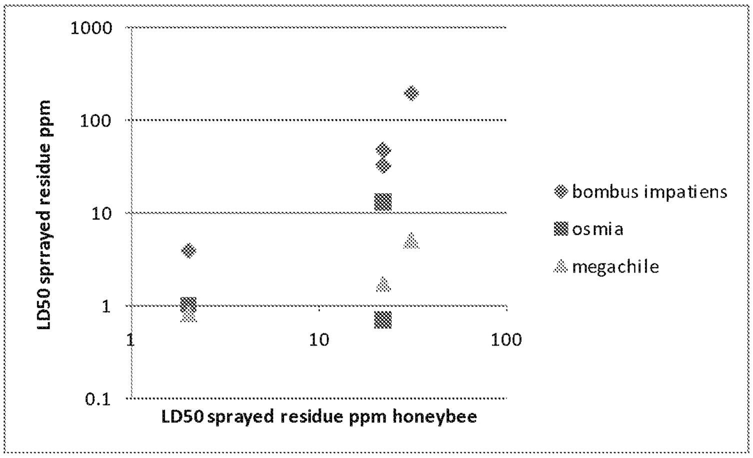

Figure 13. Comparison of the toxicity (LD_{50}) of sprayed residues of clothianidin, imidacloprid, *lambda*-cyhalothrin and spinosad to adults of *Apis mellifera, Megachile rotundata*, and *Osmia lignaria* (Scott-Dupree pers comm.)

Non-Apis Bee Testing Methods

The social non-*Apis* bee species most readily manipulated in the laboratory are the Bombinae and the Meliponinae (stingless bees). Some Bombus species are also readily available through commercial sources as they are used in commercial pollination of greenhouse crops. Therefore in most regions social bees for use in studies will not require import procedures as mostly the local Bombus species are used. Several laboratory studies with non-*Apis* species have been published which reflect a range of methods (**Table 2**). **Table 3** lists non-*Apis* species for which information on culturing needs is available, however, a ready source of the bees for testing may not be available for many countries. This table also provides limited information on oral and contact toxicity test design elements used in non-*Apis* bee studies.

Table 2 Published Laboratory Tests with non-Apis Bees and Associated Methodologies

Species	Oral	Contact	Reference
Megachile rotundata 25°C Osmia lignaria 22°C 12HL:12HD	Individually housed adult bees with access to plastic ampoule containing pesticide inserted at base of periwinkle flower 87-90% success rate		Ladurner *et al.* 2003[30]; 2005[31]
Megachile rotundata held at 29°C 12 hrs light:12 hrs dark	Group feeding of 10 newly emerged bees on 1 mL	1. Direct application – held at 25°C for 20 mins to reduce activity, 1 μL applied to dorsal thorax 2. Filter paper soaked in pesticide and dried	Huntzinger *et al.* 2008[32]
Bombus impatiens, *Megachile rotundata,* *Osmia lignaria* 25°C 24HD		Contact with treated filter paper	Scott-Dupree *et al.* 2009[33]
Megachile rotundata (4-5 day old adults); *Nomia melanderi* (2-3 week old) 26-29°C,		Direct application to mesoscutum	Mayer *et al.* 1998[34]

[30] Ladurner E., Bosch, J., Maini, S., and Kemp, W.P. (2003) A method to feed individual bees (Hymenoptera: Apiformes) known amounts of pesticides. Apidologie 34 597-602

[31] Ladurner, E., Bosch, J., Kemp, W.P., and Maini, S. (2005) Assessing delayed and acute toxicity of five formulated fungicides to *Osmia lignaria* Say and *Apis mellifera.* Apidologie 36 449-460

[32] Huntzinger, C.I., James, R.R., Bosch, J., and Kemp, W.P. (2008) Fungicide tests on adult alfalfa leafcutter bees (Hymenoptera: Megachilidae) J Econ Entomol 101 (4) 1088-1094

[33] Scott-Dupree, C.D., Conrol, L., Harris, C.R., (2009) Impact of currently used or potentially useful insecticides for canola agroecosystems on *Bombus impatiens*, (Hymenoptera: Apidae), *Megachile rotundata* (Hymenoptera: Megachilidae) and *Osmia lignaria* (Hymenoptera: Megachilidae). J Econ Entomol 102 (1) 177-182

[34] Mayer, D.F., Kovacs, G., and Lunden J.D. (1998) Field and laboratory tests on the effects of cyhalothrin on adults of *Apis mellifera* , *Megachile rotundata* and *Nomia melanderi*. J Apic Res 37 (1) 33-37

50%RH			
Osmia lignaria 22°C 60-80% RH, 12HL:12HD	Individually fed using flower (cherry) method For delayed activity fed on fresh sucrose	Cooled to 4°C before dosing, 1 µL applied to thorax	Ladurner *et al.* 2005[35]
Nomia melanderi, Megachile rotundata 29.5°C, 60%RH	Placed into tubes inserted in caps of glass vials with individual bees, group housed after dosing	Direct application to dorsal thorax	Johansen *et al.* 1983[36]
Megachile rotundata 25°C constant light		1 µL applied to thorax of males and females	Tasei *et al.* 1988[37]
Bombus terrestris	Individually dosed and then group housed	1µL applied to ventral thorax	Thompson 2001[38]

Table 3. Species of social stingless bees with known culturing needs.

Species	Organizations	Reference
Scaptotrigona postica	Social Stingless bee	Nogueira-Neto, 1997[39] (Brazil)
(Melipona scutellaris	Social Stingless bee	Nogueira-Neto, 1997 (Brazil)
Melipona ferrugenea	Social stingless bee	Not documented; meliponiculturist in Kenya
Hypotrigona gribodoi	Social stingless bee	Not documented; meliponiculturist in Kenya
Meliponula bocandei	Social stingless bee	Not documented; meliponiculturist in Kenya

[35] *Ibid* Ladurner *et al.* 2005.
[36] Johansen,C.A., Mayer, D.F., Eves, J.D., and Kious C.W. (1983) Pesticides and Bees Environ. Entomol. 12: 1513-1518
[37] Tasei, J.N., Carre, S., Moscatelli, B., and Grondeau C (1988) Recherche de la D.L. 50 de la deltamethrine (Decis) chez *Megachile rotundata* F. Abeille pollinisatrice de la luceme (*Medicago sativa* L.) et des effets de doses infralethales sure les adules et les larves. Apidologie 19 (3) 291-306
[38] Thompson H.M. (2001) Assessing the exposure and toxicity of pesticides to bumblebees (*Bombus* sp.) Apidologie 32 305-321
[39] NOGUEIRA-NETO, P. Vida e criação das abelhas indígenas sem ferrão. Editora Nogueirapis. São Paulo, SP – Brasil. 445p. 1997

Non-Apis Larval Testing

Published laboratory studies conducted with non-*Apis* la rvae are more limited, these are listed in **Table X**.

Table X. Larval test methods for larval non-*Apis* bee species

Osmia lignaria 29°C 12 hrs light:12 hrs dark	Eggs raised on treated pollen in 24-well culture plates , cocoons overwintered and emerged	Timing and completion of larval development; mortality; emergence, sex and weight	Abbott *et al*. 2008[40]
Megachile rotundata 29°C	Eggs collected from leaf tunnels separated into 96-well plates and dosed pollen; cocoons overwintered and emerged	Timing and completion of larval development; mortality; emergence, sex and weight	Abbott *et al*. 2008[41]
Osmia cornuta 23°C, 70% relative humidity	Eggs placed on provisions in gelatin capsules , 1µL applied to surface of provisions	Mortality	Tesoriero *et al*. 2003[42]
Megachile rotundata 30°C, 50% relative humidity	Leaf envelope opened and provision dosed	Weight of emerged adults	Peach *et al*. 1995[43]
Nomia melanderi, *Megachile rotundata* 29°C, 60% relative humidity	Eggs and young larvae directly dosed	Completion of cocoons	Johansen *et al*. 1983[44]

[40] Abbott, V.A., Nadeau, J.L., Higo, H.A., Winston, M.L. (2008) Lethal and sublethal effects of imidacloprid on *Osmia lignaria* and clothianidin on *Megachile rotundata* (Hymenoptera: Megachilidae) J Econ Entomol 101 (3) 784-796

[41] *Ibid* Abbott *et al*. 2008.

[42] Tesoriero, D., Maccagnani, B., Santi, F., and Celli, G., (2003) Toxicity of three pesticides on larval instars of *Osmia cornuta*: preliminary results Bulletinof Insectology 56 (1) 169-171

[43] Peach, M.L., Alson, D.G., and Tepedino, V.J. (1995) Sublethal effects of carbaryl bran bait on nesting performance, parental investment and offspring size and sex ratio of the alfalfa leafcutting bee (Hymenoptera: Megachilidae) Environ Entomol 24 (1) 34-39

[44] *Ibid* Johansen *et al*. 1983.

Megachile rotundata 30°C	Male immature stages, dosed pollen provision	Number developing, cocoon completion,	Tasei *et al*. 1988[45]
Bombus terrestris	Larvae kept 10/egg cup with 3 adults 28°C, 50% relative humidity, tested 1-, 4- and 6-day old larvae, fed treated pollen dough or sucrose 24 hrs,	mortality	Gretenkord and Drescher 1996[46]

Sub-lethal effects and test developments

Sublethal effects are defined as the effects to individual that survives exposure to a pesticide. A review of sublethal effects reported in published literature has revealed insights into the effects of pesticides including effects on physiology and behavior (Desneux *et al*. 2007[47]). Behavioral effects of pesticides on bees were largely investigated in the honeybee over the last ten years. Researchers have hypothesized that foragers collecting nectar and pollen were exposed to low doses of insecticides, which caused behavioral effects and subsequently reduced homing/navigational behavior of bees (Maxim and van der Sluijs 2007[48]; Chauzat *et al*. 2009[49]). This section discusses some of the methods that have been developed to measure the potential sublethal effects of pesticides on honeybees.

[45] *Ibid* Tasei *et al*. 1988.

[46] Gretenkord, C., and Drescher, W. (1996) Laboratory and cage test methods for the evaluation of insect growth regulators (Insegar, Dimilin) on the brood of *Bombus terrestris* L. Proceedings of the 6[th] ICP-BR Symposium, on Hazazrds of Pesticides to Bees, Braunschweig, Germany

[47] Desneux N, Decourtye A, Delpuech JM (2007) The sublethal effects of pesticides on beneficial arthropods. Annu Rev Entomol; 52:81-106.

[48] Maxim L, van der Sluijs JP (2007) Uncertainty: cause or effect of stakeholders' debates? Analysis of a case study: the risk for honeybees of the insecticide Gaucho. Sci Total Environ; 376:1-17

[49] Chauzat MP, Carpentier P, Martel AC, Bougeard S, Cougoule N, Porta P, Lachaize J, Madec F, Aubert M, Faucon JP (2009) Influence of pesticide residues on honeybee Hymenoptera Apidae colony health in France. Environ Entomol; 38:514-523

Proboscis Extension Response (PER) in Laboratory

Background

When landing on a flower, the forager extends its proboscis as a reflex when the gustatory receptors set on the bee's tarsae, antennae or mouth-parts are stimulated with nectar. This reflex leads to the uptake of nectar and induces the memorization of the floral odors diffusing concomitantly. Thus, the memorization of odors plays a prominent role in flower recognition during subsequent forage trips by the same individual (Menzel *et al*. 1993[50]). The olfactory learning involved in flower recognition can be studied in laboratory with a bioassay based on the conditioning of the proboscis extension reflex (PER) (Takeda 1961[51]). Under laboratory conditions, learning and memory can be analyzed using a bioassay based on the olfactory conditioning of the PER on restrained individuals.

Principle

The classical odor conditioning of the PER is based on the temporal paired association of a Conditioned Stimulus (CS) and an Unconditioned Stimulus (US). During conditioning, the PER is elicited by contacting the gustatory receptors of the antennae with a sucrose solution (US) while an odor (CS) is simultaneously released (**Figure 1**). The proboscis extension is immediately rewarded (Reward R) by the uptake of the sucrose solution. Bees can develop the PER as a Conditioned Response (CR) to the odor alone after even a single pairing of the odor with a sucrose reward.

[50] Menzel R, Greggers U, Hammer M (1993) Functional organization of appetitive learning and memory in a generalist pollinator, the honey bee. In: Papaj DR, Lewis AC eds. Insect learning, Chapman Hall, New-York, pp. 79-125.
[51] Takeda K (1961) Classical conditioned response in the honey bee. J Insect Physiol; 6:168-179.

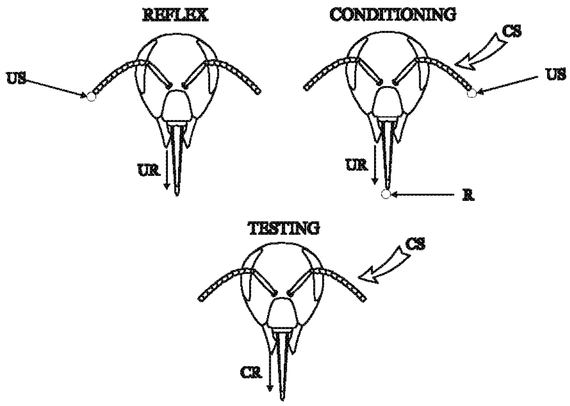

Figure 1. Conditioning Proboscis Extension (CPE) assay. The proboscis extension reflex (Unconditioned Response-UR) is elicited by contacting the antennae with a sugar solution (Unconditioned Stimulus-US). For the conditioning trials, this reflex is elicited during the delivery of odor stimulation (Conditioned Stimulus-CS). The honey bee is immediately rewarded by the uptake of sugar solution (Reward-R). During the testing trials, if the bee is properly conditioned, the delivery of the CS alone induces a conditioned proboscis extension response (Conditioned Response-CR).

Strengths/weaknesses

The PER assay with restrained workers has been used to investigate the behavioral effects of about 30 pesticides (Decourtye and Pham-Delègue 2002; Weick and Thorn 2002; Abramson *et al.* 2004; Decourtye *et al.* 2004[52]). An acute exposure to a test compound can be applied before, during, or after the PER conditioning; however, a long-

[52] Decourtye A, Armengaud C, Renou M, et al. (2004) Imidacloprid impairs memory and brain metabolism in the honey bee (*Apis mellifera* L.). Pestic Biochem Physiol; 78(2):83-92.

term exposure test scenario is more relevant to pesticide compounds with systemic characteristics. (Long-term exposure to a non-systemic compound is possible thusly; a young bee transitions to foraging after having been exposed to [non-systemic] residues via food during its in-hive life stage.) PER tests have recorded reduced learning performances for bees after 11 days of treatment with insecticides orally (Decourtye *et al.* 2003) and topically (Aliouane *et al.*, 2009[53]). The PER assay can also be used to investigate how a chemical treatment can interfere with medium-term (Decourtye *et al.* 2004[54]) or the long-term olfactory memory (El Hassani *et al.* 2008[55]).

The PER method can be used to characterize a no-observed effect concentration or lowest observed effect concentration (NOEC or LOEC). Although carried out under unnatural conditions, the conditioning of PER can provide useful information that can be related to the memory and olfactory discrimination abilities of free-flying foragers. However, there is uncertainty regarding the extent to which the laboratory PER assay reflects what may occur under more natural foraging conditions where bees are not restrained. PER testing that results in statistically significant effects on olfactory learning, should be followed up with additional testing, *e.g.*, semi-field testing using intact colonies and tests such as those described in the next section.

Artificial flowers in Semi-field Cage

Olfactory processing can be investigated using free-flying foragers visiting artificial flower feeders. The use of artificial flower feeders simulates a natural foraging situation more closely than does the laboratory tests on restrained worker bees using the conditioned PER procedure.

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[53] Aliouane Y, El Hassani AK, Gary V, et al. (2009) Subchronic exposure of honeybees to sublethal doses of pesticides: effects on behaviour. Environ Toxicol Chem; 28(1):113-122.
[54] *Ibid* Decourtye *et al.* 2004.
[55] El Hassani AK, Dacher M, Gary V, et al. (2008) Effects of sublethal doses of acetamiprid and thiamethoxam on the behavior of the honeybee (*Apis mellifera*). Arch Environ Contam Toxicol; 54:653-661.

Artificial flower experiments are performed with a nucleus ("nuc") colony (about 4000 workers and a fertile queen) placed in an outdoor flight cage. Three feeding periods are included. The initial feeding is with an untreated (blank) sucrose solution (500 $g.kg^{-1}$) delivered in both the artificial flower feeder and a standard feeder placed in the flight cage; the second feeding is treated sucrose solutions; and, the third feeding is again, an untreated (blank) sucrose solution. The foraging activity and the learning performances are evaluated using an artificial flower feeder adapted from the experimental device described by Pham and Masson (1985). The feeder consists of six feeding sites distributed on a circular gray tray (50 cm diameter). Each artificial flower feeder is a plastic Petri dish containing glass balls (allowing landing of foragers on the feeding sites) and filled with a sucrose solution that is treated or not with the test chemical. The sucrose solution in each Petri dish is maintained at a constant level, and on each side of the feeding sites an odorant (*e.g.*, pure linalool) is allowed to diffuse. To limit the influence of visual or spatial cues, the artificial feeder is rotated slowly (*e.g.*, ⅓ rpm). The device is placed in front of the hive entrance.

The conditioning (pairing odor/sucrose reward) is conducted for 2 hrs on the first day. Testing is then carried out on the following days. The testing device is set with 3 scented sites alternating with 3 unscented sites, without any food reward. The testing device is presented for 5 min and then replaced by the conditioning device for 15 min, with the odor being again associated with a sucrose solution (treated or untreated). For each observation (every 30 seconds over the 5-min observation period), the number of forager visits on either the scented sites or the unscented artificial flowers is recorded. After each test, the tray is cleaned with ethanol and the Petri dishes are changed to avoid the deposition of marking scent by the forager bees. The volume of sucrose solution up taken by the foragers is measured.

Strengths/weaknesses

The comparison of responses of honeybees before and after exposure to the test chemical on the same colony is probably the main limit of this device. Moreover, there are many unknown points, such as the reliability, the sensitivity to large panel of pesticides with various modes of action. Another uncertainty is the actual exposure to individual bees as bees are not restricted in the length of time they feed at the artificial flowers. Therefore, it is very difficult to characterize the concentration-response relationship.

Visual Learning Performance in a Maze

To test whether a pesticide compound can disorientate foragers, a maze test has been developed. Orientation performance of bees in a complex maze relies on associative learning between a visual mark and a reward of sugar solution.

The colony is maintained in an outdoor flight cage covered with an insect-proof cloth. The maze consisted of a matrix of 4 rows × 5 columns of identical cubic boxes, each side of the box measuring 30 cm; each wall has a 4-cm diameter hole in its centre through which bees can move to the adjacent box (Zhang *et al.* 1996[56]). The boxes are made of white opaque Plexiglas and a metallic screen (3 mm × 3 mm mesh) covers the maze. Bees fly through a sequence of boxes to reach a feeder containing a reward of sugar solution. The path through the maze spans 9 boxes, including 3 decision boxes and 6 non-decision boxes. A non-decision box has two holes, each in a different wall, where the bee entered through one hole and is then expected to leave through the other hole. A decision box has three holes, each in a different wall, where the bee enters through one hole and is then expected to choose between the two other holes. Finally, the forager bee is released from the box in which she was confined.

During conditioning, bees are collectively trained to associate a mark (designating the correct hole/path) with food. To that end, a same mark is fixed in front of the correct hole/path as well as the sucrose solution feeder outside the maze for one hour. For an

[56] Zhang SW, Bartsch K and Srinivasan MV (1996) Maze Learning by Honeybees. Neurobiol Learn Mem; 66:267-282.

additional hour, the feeder is placed in the first box of the path for about 30 min, then in the second box of the path the next 30 min, then in the third box during for 30 min and so on. The feeder is then moved to the fifth box for about 20 min. Finally, the feeder is placed at the end of the path (Figure) in the reward box. Several conditioning periods (3-5) are necessary to train a sufficient number of bees. After the bees have found the food (reward) and have fed, the bees are captured on the sugar syrup feeder and are then placed in rearing cages equipped with a water supply and a sugar syrup feeder (50 % w/w). The bees are put back into laboratory and kept at a temperature of 25 ± 2°C in artificial light while they are labeled with colored and numbered tags.

For oral delivery, the treatment chemical is added to a sucrose solution (50% w/w). The effect of the treatment solution on performance is then compared with that of an untreated sucrose solution. After 1.5 - 2hrs of starvation period, each group of tagged foragers receives a volume of the treated sucrose or the control sucrose solution, during daylight and at 25 ± 2°C. The volumes are adjusted for a consumption of syrup estimated to be approximately 10 µL per bee. After complete consumption of the sugar solution, a new starvation period of about two hours is initiated. Then, the bees are provided with an untreated sugar solution *ad libitum* and the bees are then released at the hive entrance.

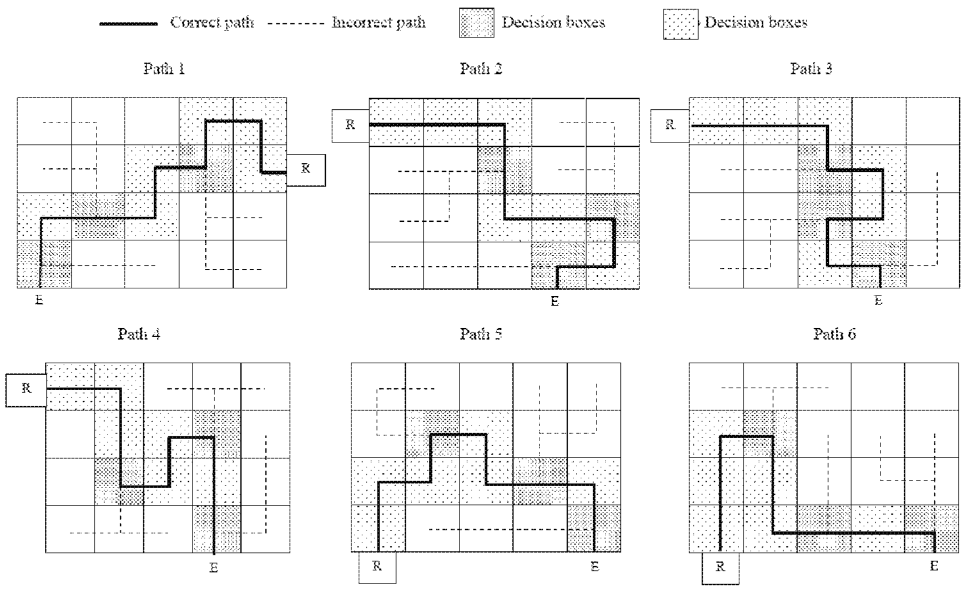

Figure 15. Maze paths used before, during and after treatment. Path 1 is used for the conditioning procedure and other paths are used for the retrieval tests. Each path started with the entrance (E), contained 3 decision boxes, 6 no decision boxes, and finished with the reward box (R).

After conditioning, the capacity of an individual bee to negotiate a path through the maze is tested. An observer notes the number of correct and incorrect decisions, and then number of turns back. During retrieval tests, several different paths are used. During a test, only one bee is allowed into the maze at a time and she is tested for one of the five path configurations.

Four categories of performances are defined and one of categories is assigned to each of them:

1. bee moves through the maze and arrives directly at the goal (reward box);
2. bee moves through the maze and arrives to the goal with one or more turns back (bee leaves the box through the hole from which it entered);
3. bee moves through the maze with mistake(s) (bee making one or more wrong turns at the decision boxes) but arrives to the goal;

4. bee does not arrive to the goal within 5 min after entering the maze.

Performances of control and treated bees are evaluated as the mean of the categories assigned to bees in each group. The time required to reach the goal from the instant of entering the maze is measured for each bee. Flight time is considered only for bees flying through the whole path within 5 minutes.

Strengths/Weaknesses

Menzel *et al*. (1974)[57] have demonstrated that honeybees in flight can associate a visual mark to a reward and, this associative learning is used by bees to negotiate a path in a complex maze (Zhang *et al*. 1996[58]). After treatment with a sublethal dose of a chemical, the ability of bees to perform the task can be impaired compared to untreated control bees (Decourtye *et al*. 2009[59]). Studies have shown that orientation capacities of foragers in a complex maze can be affected by a pesticide. The maze test relies on the visual learning of foragers in relation to navigation. However, while the maze test has demonstrated effects with pesticides which are neurtotoxic, there are insufficient data at this time to determine whether the test will provide useful information for chemicals with other modes of action. Additionally, bee navigation in the field relies upon several guidance mechanisms, (*e.g*., position of sun, magnetism, *etc*.). unlike in the maze where performance is based on the use of a limited number of pertinent cues. Additional experiments are needed to establish whether effects on maze performance reflect what may actually occur when foragers exposed to pesticides in the field and are then confronted with complex environmental cues.

[57] *Ibid* Menzel *et al*. 1974.
[58] *Ibid* Zhang *et al*. 1996.
[59] *Ibid* Decourtye *et al*. 2009.

RFID Tagged Bees to Measure Foraging Behavior

Background

Experimental test situations have been designed in relation to feeding behavior and social communication (Schricker and Stephen 1970[60]; Cox and Wilson 1984[61]; Bortolotti *et al.* 2003[62]; Yang *et al.* 2008). These studies recorded the trips between a feeder and a hive, and the bees were captured on the feeder and marked with paint or colored number tags. These techniques are limited by the number of individuals that can be simultaneously monitored, and in the time devoted to recording individuals. To address this limitation, automated tracking and identification systems have been developed using radio frequency (RF) transponder technology. The use of transponders has the potential to revolutionize the study of insect life-history traits, especially in behavioral ecotoxicology.

Different transponder devices have been employed on the honeybees: harmonic radar (*e.g.*, Riley and Smith 2002[63]) and Radio Frequency Identification (RFID; Streit *et al.* 2003[64]). Currently, the RFID tags seem to be the technology offering the most advantages. Advantages include the large number of individual insects that can be tracked, the number of detections which can be monitored rapidly and simultaneously (milliseconds) without interference from a variety of matrices (*e.g.*, propolis, glue, plastic, wood, *etc.*) frequently encumbering visual observations, and less disruptive effect on bee behavior given the small size of the RFID tags compared to what is needed for harmonic radar tracking.

The tag itself is not equipped with a power source (passive function); rather, it obtains its signal power from the detector (transponder) and causes the tag to emit a unique

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[60] Schricker B, Stephen WP (1970) The effects of sublethal doses of parathion on honeybee behaviour. I. Oral administration and the communication dance. J Apicult Res 9:141–153.

[61] Cox R. .L and W. T Wilson (1984) Effects of permethrin on the behavior of individually tagged honey bees, *Apis mellifera* L. (Hymenoptera: Apidae). Environ Entomol; 13:375-378.

[62] Bortolotti L, Montanari R, Marcelino J, Medrzycki P, Maini S, Porrini C (2003) Effects of sub-lethal imidacloprid doses on the homing rate and foraging activity of honey bees. Bull Insectol; 56:63-67.

[63] Riley JR, Smith AD (2002) Design considerations for an harmonic radar to investigate the flight of insects at low altitude. Comput Electron Agric 35:151–169.

[64] Streit S, Bock F, Pirk CWW, Tautz J (2003) Automatic life-long monitoring of individual insect behaviour now possible. Zoology 106:169–171.

identification code. The detector (reader) can recognize a virtually unlimited number (18×10^{18} possible identification codes) of individually tagged insects. The RFID technology allows detecting each time a tag-equipped bee is passing in close proximity to the reader (working distance of approximately 3 m)n a study to determine the error rate, Streit *et al.* 2003[65] demonstrated that 1 out of 300 tagged bees was not recorded by the RFID readers.

Experimental Procedure

Using this test technology, the experimental colony is maintained in an outdoor tunnel (8 m × 20 m, 3.5 m high) covered with an insect-proof cloth and the ground covered with a double layer of plastic. Bees are fed with pollen which is renewed daily. A sucrose solution (50% w/w) is delivered by a feeder positioned 18 m from the hive entrance, in a wooden box (26 cm × 26 cm, 30 cm high).

RFID tags (64-bit, 13.56 MHz system, 1.0 mm × 1.6 mm × 0.5 mm), weighing about 3 mg (3% of bees' weight), represent a relatively low weight given that the honeybee is able to carry up 70 mg of nectar (Ribbands 1953[66]) and 10 mg of pollen (Hodges 1952[67]). A tag-equipped bee passing underneath the reader is identified by the reader that sends the data along with real-time recording to a database. Five readers are placed at the entrance of the hive and the artificial feeder which serve as the recording points (five readers per recording point, with a total of ten readers. By passing underneath the reader both at the hive and at the feeder, the foraging bee is monitored twice, thus determining the direction of target and the travel time between the two recording points. The reader software records the identification code and the exact time of the detection automatically for 6 days in a database for later analysis of spatial and temporal information. Such analyses may include time spent within the hive, the time spent at the feeder, the time

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[65] *Ibid* Streit *et al.* 2003.

[66] Ribbands CR (1953) The behaviour and social life of honeybees. London Bee Research Association, London.

[67] Hodges D (1952) The pollen load of the honeybee. London Bee Research Association, London.

spent between the feeder and the hive, the number of entries into and exits from the hive, and the number of entries into and exits from the feeder.

Strengths/Weaknesses

RFID devices allow the study of both the behavioral traits and the lifespan of bees, especially under biotic and/or abiotic stress. However, the large quantity of data obtained with this technique requires an interface for analyzing the data and providing the life-history traits of individual bees. Under semi-field conditions, RFID microchips have provided detectable effects due to exposure to an insecticide (Decourtye *et al.* 2011[68]).

Conclusions

Although laboratory toxicity tests are currently available for evaluating the potential effects of chemicals on bees, there is no single consistent approach used by different regulatory authorities and, therefore, the design and scope of these tests vary. For the purposes of screening-level risk assessments, many regulatory authorities rely on acute toxicity tests using young adult honeybees and these tests may only evaluate contact toxicity although acute oral toxicity test guidelines exist. While guidelines are becoming available that include acute toxicity tests with honeybee larvae, there is also need to expand these laboratory test methods to examine the effects of chemicals from subacute and chronic exposure durations. Laboratory-based studies will likely continue to focus on individual test organisms; and although laboratory-based toxicity testing has historically focused on frank mortality, tests are evolving to provide insight on sublethal effects such as impaired behavior. As the range of measurement endpoints continues to expand, there is a need to provide both qualitative and quantitative linkages between measurement endpoints and assessment endpoints on which regulatory authorities

[68] Decourtye A, Devillers J, Aupinel P, Brun F, Bagnis C, Fourrier J, Gauthier M (2011). Honeybee tracking with microchips: a new methodology to measure the effects of pesticides. Ecotoxicology; 20:429-437.

typically base decisions. Efforts are also underway to expand the range of test species to address concerns that the *A. mellifera* may not be an adequate surrogate for non-*Apis* bees with considerably different life cycles.

CHAPTER 9 ASSESSING EFFECTS THROUGH SEMI-FIELD AND FIELD TOXICITY TESTING

Introduction

Semi-field and field studies may be conducted for regulatory purposes if lower tier assessments trigger further evaluation of a chemical's potential to cause adverse effects. For example, a regulatory trigger value may have been breached in the lower tier assessment which in turn means that a protection goal may not be met based on the findings at that level. One way to ensure that the protection goal is met is to modify the use of the subject compound such that it may no longer pose an unacceptable risk to the honey bees *Apis mellifera*[69] and/or non-*Apis* bees[70]. However, modifying or restricting the use of a compound may be undesirable or unnecessary if further information is obtained from either a semi-field or field study that demonstrate otherwise. Such a study or studies should provide greater insight into whether adverse effects to *Apis* and/or non-*Apis* bees are likely to occur under real-world field use of the pesticide in question. As such, the objective of the regulatory study(ies) may be to try to indicate, both quantitatively and qualitatively, what the possible effects may be under more environmentally realistic or relevant conditions. Such studies should be predicated on well developed problem formulation that build on lower-tier studies as well as the associated risk assessment. As part of the problem formulation there should be clear identification of the protection goals, assessment endpoints for determining whether protection goals have been met and measurement endpoints used to examine assessment endpoints

This chapter provides an overview of what to consider when planning or assessing either a semi-field or field study. As regards the honey bee, much use has been made of EPPO

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[69] It should be noted that when referring to *Apis mellifera*, we are referring to the approximately 17 subspecies that originated in Europe.

[70] Non-*Apis* bees are highly varied in terms of social and solitary lives, the duration of their activity in the field, the amount of pollen and nectar they store, and where they nest. For details, see Chapter ####.

170[71] and OECD 75[72]. Participants during the SETAC 2011 Workshop used their own practical and regulatory experience to provide further information on how a study should be conducted. Therefore, the following is seen as a development of both EPPO 170 and OECD 75 based on the experience of the experts present at the workshop. If the risk assessor indicates the need for either a semi-field or field study, then it is recommended that both this Chapter along with information provided in EPPO 170 and OECD 75 be consulted. The information in these references may also be consulted when such studies are being evaluated for regulatory purposes.

Definition of Semi-field and Field Studies

Elements in the design of semi-field and field studies encompass the study's objectives, the test organism, a study site, methods, endpoints, sample design, quality assurance/quality control standards and the statistical analysis of the data. In discussing the elements of a semi-field study, the Participants of the Workshop defined a semi-field study as the following:

> A **semi-field study** is designed to measure exposure and/or effects and is performed on a crop that is grown outdoors in an enclosed test system with controlled or confined exposure. The crop is subject to good agricultural practices (*i.e.*, grower standard practices), and therefore, there will or could be weeds present but the predominant plant, and thus the source of nectar/pollen, will be the crop. The test system could, nevertheless, be designed to reflect a desired exposure system and specific foraging environments, *i.e.*, a mixture of crop and weeds, flowering margins, etc. The details of the test design (application parameters, measurement endpoints, etc.) will depend upon the regulatory question(s) being asked. However, semi-field studies generally attempt to

[71] [HYPERLINK "http://onlinelibrary.wiley.com/doi/10.1111/j.1365-2338.2010.02418.x/pdf"]
[72] [HYPERLINK "http://www.oecd.org/officialdocuments/displaydocumentpdf?cote=env/jm/mono%282007%2922&doclanguage=en"]

maximize exposure by confining bees to a particular source of treated nectar/pollen.

For species (non-*Apis* species and *Apis* species) that are used to pollinate plants grown in glasshouses it may be necessary to carry out a higher tier study. A semi-field study will be enclosed with controlled or confined exposure but be of reduced size compared to a commercial glasshouse. Size of the test environment is related to the species being studied, and the questions or issues being investigated.

A semi-field study, therefore, provides for a potentially worst-case exposure scenario (see Section 1.4.4 for further information on this point).

A **field study** is designed to measure exposure and/or effects and is performed on a crop that is grown outdoors with no enclosure. The crop established and maintained following good agricultural practices. The bees are free flying and able to seek out alternative food sources, however, alternative sources of pollen and nectar should be minimal (see below for further details). The study design elements (*e.g.*, selection of crop, duration of the study, environmental conditions, etc.) will depend upon the question(s) being asked. A field study for a glasshouse situation should be conducted in a commercial glasshouse.

Protection or Management Goals

As with any environmental assessment it is important to have a clear idea as to the regulatory concern or question(s) being addressed, which in turn should be based on clear protection or management goals. For purpose of developing guidance relative to higher tier tests, the participants of the Worikshop assumed that the protection goals are those proposed by the participants of the workshop and listed below (see Chapter 3 for more discussion on protection goals). The assessment endpoints of the higher tier studies are focused on ensuring that protection goals are met.

1. Protection of managed pollination in agricultural/horticultural-based crops (*i.e.*, *Apis* and non-*Apis* species)
2. Protection of honey production and other hive-products (primarily *Apis mellifera* and Meliponini only)
3. Protection of biodiversity (primarily non-*Apis* bees)

Design of a Semi-field Study

When deciding whether a semi-field study is appropriate, it is necessary to consider various strengths and weaknesses of this type of study to ascertain whether it is the most appropriate way to refine the understanding of the potential risks from the use of a compound. Outlined below are the strengths and weaknesses of semi-field studies for *Apis* and non-*Apis* bees.

Apis mellifera

Strengths
Exposure is known to have occurred as the bees are enclosed and there is usually a toxic standard test group, *i.e.*, the toxic standard reference chemical is used to confirm that the bees are exposed to the treatment and to calibrate the ability of study to detect treatment effects known to be associated with the reference chemical.
Provides realistic exposure both inside and outside the hive, i.e., to both material available at the target crop, as well as concentrations in the hive.
The test system can also be designed to determine the residual toxicity. Weathering of the applied material and natural exposure of honey bees is inherent in the design.
Irrigation of the crop (via drip irrigation to avoid wash-off) is possible, hence potentially reducing the likelihood of the study being adversely affected by drought.
In contrast to laboratory studies, semi-field studies present a more realistic scenario of

Strengths
interaction between the bees and the environment.
Due to their smaller size and shorter duration semi-field studies are less affected by fluctuations in physiological background, and ecological variables.
Potential for sub-lethal effects can be observed more easily than in either laboratory or field studies.
Brood can be considered in specifically designed semi-field studies (see OECD 75).
Semi-field tests are relatively quick and easy to perform.
Semi-field environments are smaller-scale in operation than field studies, making it feasible to test greater numbers of replicates, which in turn should allow for more robust statistical designs.
As the bees are enclosed and have no alternative foraging environment, the exposure is potentially a "worst-case" scenario.
Certain exposure scenarios that are difficult to study under real field conditions, e.g. aphid honeydew, can be studied under semi-field conditions.

Weaknesses
Experience with performing these studies has shown that it is difficult to keep colonies in an enclosed structure for long periods; and as a result there is a limited amount of time that a colony of *Apis mellifera* can survive in the enclosure. The correct stage of crop bloom is critical to the study and as a result, it is only appropriate to assess the effect of short-term exposure including potential effects on brood (see OECD 75).
Semi-field studies tend to use colonies with only 3,000 – 5,000 bees (EPPO 170), which is smaller than a full size [managed] colony. Hence, extrapolation of adverse effects to a full size unenclosed colony under more realistic field conditions, may not be possible.
Due to the small size of the colony it is not as easy to assess pollen and nectar storage and hive weight development; therefore, it is difficult to assess potential effects on

Weaknesses
honey production (i.e. a potential protection goal) when adverse effects are observed on other parameters.
Semi-field studies may not provide information on overwintering success.
Due to the nature of the enclosed test design, not all crop scenarios are possible to test, (*e.g.*, size of plants, area required, and nutritional value of crop to bees)
There is potentially limited foraging area; therefore, care is needed to ensure that sufficient area (nutrition) is available.
There is a possible stress on bees due to enclosed nature of the study, *i.e.*, bees have a desire to escape, consequently reducing their foraging activity on the crop. However, balance of tent size/crop field size and colony size should ensure foraging and exposure (see EPPO 170).

Non-*Apis* bees

Strengths
Individual colonies (in the case of Meloponini (stingless bees) or *Bombus*) or aggregations of individual solitary bees are used and thus the pesticide effects are readily interpreted. Increased replications are possible and readily performed so statistical analysis may be easier.
Product use on a wide range of crops, including those that are not readily pollinated by honey bees (*e.g.*, eggplant), can be assessed.
Some social non-*Apis* bees such as Meloponini (stingless bees) and *Bombus* are easier to handle than *Apis* as they are reluctant to sting. Additionally, many of the solitary non-*Apis* bees although capable, are reluctant to sting. Solitary bee species amenable to semi-field studies (*e.g.*, *Osmia* and *Megachile* species) will not sting.
The area of the enclosure of a semi-field study can support full colonies if non-Apis species (*Bombus* or Meloponini) or a collection of independent individuals (solitary bees), hence an extended study can be done. These bees have a complete life cycle in

three to six weeks (solitary bees) or season (*Bombus*) in temperate climate.
Individual solitary bees typically provision nests over a three to six week period, thus allowing for a complete (or at least almost complete) life-cycle study for solitary bees if the forage crop flowers for more than three weeks.
It is possible to do larval exposure tests with solitary bees because pollen/nectar is brought straight to a cell and an egg is laid on this. This leads to a potentially conservative assessment since the progeny has direct exposure, dermal and oral, with food resources that potentially contain the test pesticide.
Non-*Apis* bees can be used and maintained efficiently in small enclosures.
Non-*Apis* bees will forage under less optimal conditions in terms of temperature, relative humidity, and wind. This is especially true for *Osmia* and *Bombus* spp. which are quite hardy.
In solitary species such as those in *Megachilidae*, the larvae are in direct contact with nectar and pollen, and so there is the possibility of contact and oral exposure. This is not the case with *Apis* larvae that require a special larval test to expose larvae to a given pesticide and route of exposure.

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Weaknesses
Resource supplements may be needed for crops that do not provide both pollen and nectar, which may reduce bee activity.
In temperate areas, the annual life cycle of solitary bees limits the window in which adult or larval testing may be conducted.
There is significant uncertainty as to how representative the current commercially available non-*Apis* bees are for other non-Apis species. For all non-*Apis* bees, there is enormous variation in use of resources, behaviour, habitat requirements, life cycles, etc.
Many solitary bees are univoltine in temperate climates thus impossible to use all year round.

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When would a semi-field study be appropriate?

Consistent with the tiered approach to toxicity testing and risk assessment, semi-field studies may be triggered when lower tier assessments (relying on laboratory results) indicate potential risk that are inconsistent with protection goals. In such cases, higher tier tests may provide information that reduces the uncertainty about risk, allowing for a more informed decision. Outlined below are scenarios when a semi-field study may be appropriate; and where this is not the case, alternatives are proposed:

- If, as a result of the initial lab assessment, acute mortality and sub-lethal effects are considered to be the main concern, then a semi-field study may be appropriate.

- If repellency effects or an impact on foraging activity is predicted, either on the basis of efficacy data (e.g. a compound is known to act via an anti-feedant effect) or from any observations from either laboratory studies or any other relevant studies; then a semi-field study may give the risk assessor useful information on the potential short-term effects the compound may exert on foraging behaviour. Due to the confined nature of the study, it can be concluded that no effects in a semi-field study probably equates to no short-term effects under field situations. However, if an effect on foraging behaviour is observed, then there could be long-term effects and it may be necessary to extend the semi-field study (see 1.6.1) or conduct a full field study.

- A semi-field test may be used to validate or test a safe re-entry time for bees. Based on information gathered from a foliar residue toxicity study, (see Chapter X) a semi-field test can be used to define the use to determine the time required for the residue to become 'safe' to foraging bees. For example, a semi-field study may indicate that the residues are 'safe' to bees after 6 hours, therefore the product could carry an instruction to only apply at night, hence allowing sufficient time for the residue to dry and hence become 'safe' to bees.

- If a pesticide is systemic and to be used as either a seed treatment, solid formulation (*e.g.*, granule or pellet) or soil treatment, then a semi-field study can provide detailed information regarding exposure levels both in the target crop and in the hive associated with that specific application method. Care is required in selecting of a study site to ensure that environmental conditions (e.g., soil conditions (moisture, pH etc), duration from soil treatment to drilling and flowering) are appropriately representative of the proposed use. The study can also provide an indication of the likelihood of initial mortality and initial behavioural effects following exposure. As confinement may affect bee behaviour *per se*, it is necessary to compare effects seen with those observed in the control. If there is a possibility of long-term effects resulting from this type of exposure, then it may be possible to modify this study appropriately (see 1.6.1) or alternatively it may be preferable to conduct a field study.

- If the compound is, or exhibits insect growth regulatory characteristics, then a test according to Oomen *et al*. (1992) or a semi-field study over a 28-day period (OECD 75) can provide information on the potential effects.

One of the advantages of a semi-field study, in comparison to a field study, is that it allows for the inclusion a toxic standard (i.e., one replicate is run with a test material that is known to elicit adverse effects to the test organism). However, since there are occasions where where it is not possible to use a toxic standard (*e.g.*, systemic seed treatments[73]), the absence of a toxic standard does not greatly compromise the utility of the test. (When testing seed treatment scenarios, the residues on treated seed should be determined as well as residues in pollen and nectar; exposure to the bees is assumed as the test system is closed and exposure is compulsory.

[73] The lack of a toxic standard for a systemic seed treatment or solid formulation is due to the lack of a compound that causes known effects.

- Semi-field studies are useful studies for non-*Apis* species such as *Megachile rotundata* as they may provide information on alternate routes of exposure, i.e., leaves which are used for nest building, in addition to conventional routes of exposure such as nectar and pollen.

- It is possible to determine colony effects in a semi-field study over an extended period (*e.g.*, for three months or longer) with species such as stingless bee colonies and bumble bee. For example, a bumble bee colony may be housed in a box with two connected chambers (one chamber for the colony's nest, and one chamber from which the colony may be fed (Kearns & Thompson 2001). The nest box may be opened and the colony allowed to forage outside in a semi-field enclosure. After this exposure period, the nest may be closed and the colony fed in the nest box's feeding chamber for a month or two to look at delayed lethal or sub-lethal effects on reproduction and colony growth. After a couple of months, bumble bee colonies will switch from raising workers to raising drones and the colony will dwindle. Similarly, one can expose foragers from a stingless bee colony for several days in a semi-field enclosure and then close up the nest box. The colonies in this case can then be fed by placing food (sugar water and vitamins) at regular intervals into the nest box. Stingless bees have perennial colonies (much like honey bees) and may be fed en situ for many months.

From the above, it is clear that semi-field studies address mortality from short-term exposure as well as short-term behavioural effects; however, there is a concern whether they are able to address:

- long-term effects from either short-term/sub-lethal exposure or
- long-term effects from long-term/continual (*i.e.*, via hive products) exposure or long-term chronic exposure.

Outline of a semi-field study for *Apis* and non-*Apis* bees

Design of a semi-field study for Apis bees

The following is based largely on EPPO 170 (2010) and OECD 75, should be seen as an extension to both of these guidance documents, and should be considered along with the details of either these guidances. In developing the elements of this chapter the Workshop Participants relied upon their experience as well as information included in EPPO 170 and OECD 75. The aim of the following section is to highlight further issues to consider when planning and carrying out a semi-field study as well as issues that should be considered when evaluating a semi-field study for risk assessment purposes.

It is important that the aims of any semi-field study are clearly determined and stated (insert x-ref problem formulation chapter). Clear problem formulation is required to ensure that the study is appropriately designed and focused to address the regulatory question(s) being asked. As all semi-field studies will be designed to address specific concerns highlighted at lower tiers, they will be to some extent, bespoke in their design. EPPO 170 and OECD 75 are relatively flexible guidance documents and consequently allow studies to be designed to address specific issues. The considerations of the participants of the workshop, and of this chapter, does not remove or reduce that flexibility, it simply highlights areas or study design elements that are thought to be important considerations for incorporation into a semi-field study.

Size of Semi-field Study

The minimum size of a semi-field study enclosure according to EPPO 170 is 40 m^2. This area is recommended in EPPO 170 and is based on professional experience and is considered appropriate in terms of practicality of actually conducting the study and for determining effects of mortality and behaviour. However, this area is only appropriate in terms of certain field crops (*e.g.* *Phacelia*, oilseed rape/canola, mustard). For other crops (*e.g.* melons, apples) the area (40 m^2) may need to be amended due to issues such as the number, density and attractiveness of flowers, availability of nectar and pollen or the size of the plants. The area of the test enclosure may also need to be amended depending upon the size of the colonies being used.

It should be noted that when studying bee brood, an increased [enclosed] crop area (> 60 m^2) may be preferable to ensure the colony has access to adequate floral resources. This recommendation is based on practical experience from conducting this type of study. However, the precise area depends on colony size, crop, and duration of confinement; 40m^2 (OEDC 75) may be acceptable for a small colony that is confined for no more than 10 days.

Crop

The standard crops (*i.e.*, oilseed rape/canola, mustard and *Phacelia*) are easy to cultivate and manage but more importantly are highly attractive to honey bees. *Phacelia* has an open flower that it is highly attractive. The openness of its flower will mean that bee parts of the flower will be fully exposed to the spray application; hence, honey bees foraging after the spray application will be exposed to residues. Oilseed rape or canola and mustard are both highly attractive to honey bees hence a high level of exposure can be ensured. Results from studies carried out on these crops can be extrapolated to other crops, provided that the application parameters in terms of application rate, timing of applications and number of applications used on the surrogate crop(s) is comparable (ideally identical) to that of the subject product. If effects are observed on these standard crops then it may be possible to further refine the assessment by using the target crop species.

When considering systemic soil or seed treatments, it is preferable to use the actual/relevant crop. A crop other than the target crop may be justified on the basis of exposure (*e.g.*, it may be appropriate to select a crop that is attractive and has high residues in nectar and pollen as a 'model' crop rather than the actual crop of concern).

Size of Colony

Each tunnel/cage/tent should include one, healthy queenright (*i.e.*, a fertile, laying queen) colony per cage. Precise size of the colony used will depend upon the study design, EPPO recommends a size of 3,000 – 5,000 bees.

It is important to have sufficient nutritional resources within an enclosure to ensure that the bees are not starving. Generally feeding will not be necessary, however, if there is concern regarding the attractiveness of a specific crop/situation, then supplemental feeding may be needed. For example, if testing maize, then additional food will be required as maize produces no nectar.

Test Treatment

Sprays Only

Test treatment(s) and water (negative) controls are required; ideally a positive control (reference standard) is also required. It is customary to test the proposed field rate only. If, however, a model crop is being used, *e.g.*, *Phacelia*, then it may be appropriate to have more than one treatment rate. This may enable the data to be extrapolated to other crops and other application rates. Additional tunnels/cages could be used to address different application rates as well as effects from treating at different times of the day. However, at a minimum, a study at the maximum proposed rate should be carried out. Further details on how the results can be analysed and interpreted are provided in the chapter on statistical analysis (Ref Stats chapter).

A positive control provides: *(i)* an indication of the sensitivity of the test system; *(ii)* demonstrates exposure; and, *(iii)* indicates the magnitude of response to a known toxin. However, positive controls kill bees unnecessarily and can add to the cost and complexity of study design; therefore their use should be considered carefully. Positive control compounds are useful if it is unclear if any dose of the tested pesticide will have effects. If a positive control is used, it is necessary to select a compound whose toxicity profile is known and consistent with that under consideration, *e.g.*, for assessment of a potential acutely toxic compound, then there is a need to use a similar compound. Historically, dimethoate has been used as a reference chemical when studying acutely toxic compounds on adult forage bees. If insect growth regulatory effects are expected then a known insect growth regulator with similar effects should be used. When a positive control is used, there should always be clear effects. There should not be sustained mortality at high levels in the water control. There should be an appropriate number of replicates for the treatment group(s) to provide sufficient power to discriminate treatment effects with a level of precision.

For systemic solid formulation/seed treatments/soil treatments

While there may be desire to have both the treatment and a water (negative) control, currently it is not possible to identify a suitable positive (reference) standard for most systemic solid formulation/seed treatments/soil treatments.

Pre-application

Sprays Only

Healthy colonies should be used and transferred to the test site a minimum of 2-3 days prior to treatment. This is due to mortality that inevitably occurs when a colony is moved. If the hive is moved during the day, the hive will tend to acclimate quicker. There should be a measurement of mortality over the acclimation period; the greater number of measurements of mortality will provide greater confidence that effects after treatment are attributed to the treatment rather than due to the hive acclimation. It is likely that there will be variability between colonies and every effort should be made to ensure that they are as consistent as possible. This can be partly be achieved by moving the colonies at the same time. Attempts should be made to make sure that the colonies are as similar as possible, in terms of number of bees, at the start of the study. Excessive variation at the start of the study will make the study difficult to interpret and hence potentially limit its usefulness. (Ref Stats chapter).

Further work is required to determine the range of background levels of mortality once the colony(ies) are situated at the test location in the in order to establish acceptable levels or ranges of mortality. These background levels could be used to help interpret whether the level of mortality observed in the treatment is treatment-related or not (providing an indication as to the overall reliability of the study). Until such data are available, statistics should be used in interpreting the results (Ref Stats chapter).

With spray treatments the colony is placed in the semi-field setting when the crop is just about, or at flowering. The effects of the pesticide to honey bees foraging that crop are then determined. With systemic chemistries, exposure will occur over a longer time, therefore, the honey bees should be present during the whole flowering period of the plant. Acclimation as outlined above is, therefore, not possible as exposure of the bees to the pesticide will occur as soon as they are introduced in to the treatment area. However, a consideration of mortality due to moving the colony is still required. One potential way around this is to compare the mortality that occurs on the untreated crop to that in the treated crop. Nevertheless, the significance should be determined statistically (Ref Stats chapter).

Semi-field studies may be most effective for determining acute effects related to systemic chemistries. If sublethal effects are predicted, then a modified semi-field designed to acertain any long-term effects, or simply a full-field test may be more appropriate (see below for details).

Post-treatment assessments

Assessments of mortality via the placement of dead bee traps, sheets or tarps at the front of the hive and within the enclosure should ideally be carried out daily but at least on days 0, 1, 2, 4 and 7 post-treatment. This frequency is not appropriate for in-hive assessments as the disturbance could cause significant effects.

Sub-lethal Behavioural Tests

There is a need to standardize and refine the number and type of tests or observations that can be made to document potential behavioural changes due to sub-lethal pesticide exposure. It is typical to report that "no abnormal behaviour in foraging or other behaviours occurred during the test" but definitive and meaningful quantifiable measures are often lacking. Rather than making general observations on bee behaviour during the test period, it is proposed that more detailed observations and or measurements be made in addition to the general observations used to date. Of these perhaps the most obvious is in measuring foraging activity.

When measuring forage activity, the number of returning foragers should be counted pre-treatment and at regular intervals post-treatment. The number of returning foragers with pollen loads should constitute a separate count from those returning without pollen (nectar and water foragers). Observations should last for

1 – 3 minutes. The observation periods should be equally divided across all test groups so that measurements are taken at approximately the same time with the controls as with treatments.

A second observation that could be quantifiably measured in a semi-field test is the average flower handling time. This measure is made by recording the time taken for the bee to work a flower (i.e., to remove pollen and/or nectar). The observer simply records the total flower handling time for bees collecting pollen and nectar. If flower type is such that distinct pollen and nectar foraging is possible then these forager types should be kept separate. The exact number of measures required should be determined or justified statistically. The time of day for measurements to be taken should be randomized between plots to avoid time of day and or weather bias. As with previous studies of this type, general observations of any unusual bee behaviour should be noted and quantified if possible (*e.g.*, 30 bees were seen twitching and exhibiting excessive grooming on the landing board during the 1-3-minute foraging counts). In addition, it may be possible to determine foraging behaviour in front of the bee.

Due to the confined flight areas for bees in semi-field studies, the significance of any behavioural effects should be interpreted with caution.

Due to the confined nature of semi-field studies, it was the consensus of the Workshop Participants that an adverse effect on behaviour compared to the control should be interpreted with caution and should trigger additional consideration. The relevance of an effect, or lack thereof, in a semi-field study ~~could~~ may not be assumed to be relevant at the field scale. Interpretation of effects, or lack thereof, must be done with care. Additional information could be obtained to aid interpretation of any effects seen. This information could come from a variety of sources, however the Workshop Participants considered that field studies were the most appropriate source to validate any effects or lack of effects that are considered significant.

Depending upon the regulatory question being asked, it may be necessary to determine residues in fresh pollen, stored pollen, nectar, honey, and wax. The type(s) of samples to be collected depends on the study and the questions to be answered. Residues in foraging honey bees may also be ascertained and this information could be used in interpreting potential incidents.

Results

Traditionally when determining if a study is acceptable, there is consideration of whether it has met various quality criteria, such as adequate controls, chain of custody, etc.. In addition, there should be a consideration as to how the study compares to the above guidance. The use of a positive standard (reference chemical) can help meet the need for quality assurance measures, however, it is not essential for the reasons stated above.

Key outputs from a standard semi-field study could be:

Mortality in the crop: use of sheets or tarps in the crop.
Mortality at the hive: use of dead bee traps or sheets in front of the hives.
Foraging activity and other behaviour: see discussion above.
Measures of exposure: residues in pollen, nectar, pollen pellets, and dead bees.
Pollination deficit: it may be possible to determine if there is a difference in the degree of pollination success (*e.g.* via fruit set) of the treated versus untreated crop. See xxxx for information on measures of fruit set.
Assessment of the brood (including an estimate of adults, the area containing cells, larvae and capped cells). If this was a key area then OECD 75 should be consulted. (An actual data set, including brood counts, is provided in the Statistics chapter (Ref Stats chapter).)
Effects to brood: methods outlined in OECD 75 should be followed.

Design of a Semi-field Study for Non-*Apis*

At present there is no equivalent EPPO 170 or OECD 75 guideline for using non-*Apis* bees in semi-field or field studies. As a result, the Workshop participants suggest that if there is a regulatory question regarding a pesticide that requires the inclusion of a non-*Apis* species as a result of triggers activated by laboratory effects bioassays, the study design should be developed on a case-by-case basis with consideration of the specific endpoints described for semi-field honey bee studies and the overall regulatory question. Care should be taken when evaluating and interpreting results from these studies until protocols are sufficiently vetted through ring-testing.

When selecting non-*Apis* species to be used for semi-field studies, attention needs to be paid to their availability, ease of handing and survival under experimental conditions. Therefore, it is recommended that the species used are those that are either commercially available or can be readily reared under laboratory conditions.

Appendix X provides several draft protocols that could form the basis of semi-field studies of non-*Apis* bees conducted to address specific regulatory questions.

Semi-Field Studies - Solitary Bees

Three solitary non-social bee species are recommended for use in semi-field studies in temperate zones: *Osmia lignaria*, *O. bicornis* and *Megachile rotundata* (Johansen *et al*. 1984; Tasei *et al*. 1988; Ladurner *et al*. 2008; Konrad *et al*. 2009). *Megachile rotundata* will be used as the descriptive species in this section.

Megachile rotundata, the alfalfa leaf cutting bee, is a non-social Eurasian bee species that is widely managed as a pollinator of alfalfa for seed production in the U.S. and Canada, and is occasionally deployed for the pollination of other specialty crops (*e.g.*, canola, carrot – for seed, blueberries). Dormant alfalfa leaf cutting pre-pupae are sold as loose cells in 4 L (gallon) increments (approximately 10,000 individual cells).

Due to standard field production cycles, dormant loose cells are usually only available from late fall through early winter. Cells should be maintained at 1.7 to 4.4°C and 50% relative humidity until natural emergence during early summer in most of the northern hemisphere. Bees maintained in cold storage beyond this point begin to deplete stored energy reserves and may fail to emerge upon incubation (210 total days is the general upper limit for diapause before viability declines significantly). Cells should be stored in open or ventilated containers, and tumbled periodically to reduce the growth of moulds. Bees can be incubated to adulthood with as few as 150 days of cold storage diapause. Careful control of temperature (*i.e.*, 29°C) and humidity (70% RH) will cause most of the incubated bees to emerge from their cocoons at approximately the same time 50% emergence in 23 days and complete emergence in 32 days.

Few release rates (density rates) exist for crops with the exception of alfalfa, *i.e.*, where 74,000 to 100,000 bees per hectar are recommended, and canola and blueberries, *i.e.*, where 50,000 bees per hectar (Mader *et al.* 2010a) are recommended. Release rates will vary based on size of enclosure and crop to be utilized in the semi-field study but could be as few as 200-500 solitary bees per tunnel site of 40 m^2.

Site selection for the study should use the same criteria as those for semi-field *Apis* studies. Once an enclosure is ready, a wooden nest shelter containing enough styrofoam nesting boards to accommodate all the *M. rotundata* to be released for the study should be placed in test enclosure ~~it~~ (2 to 3 nest tunnels per bee), facing the morning sun, 3-4 days in advance of the initiation of the study (*i.e.*, before pesticide is to be sprayed in the semi-field enclosure). Bees ready to emerge or already emerged should be placed in front of the nest shelter and left to orientate to the nest. Bees should not require supplemental feed as long as there is sufficient crop in bloom. These bees do not require a water source so long as enough flowers or a nectar feeder is available. However, if mason bees (*Osmia lignaria*) are used, a drip bucket and excavated damp mud pit is needed inside a test enclosure (i.e., tunnel) cage. The mud pit should be excavated so the bees can access the soil profile layer with the best clay-water content. Nectar is not sufficient for wetting mud.

Key Outputs

- Mortality in the crop: same as for *Apis*.

- Mortality in the hive/nest shelter: use of a tarp placed on the ground in front of the nest shelter may allow some assessment of *M. rotundata* mortality. However, solitary bees may die within the nest material making mortality assessment more difficult. Assessment schedule should be the same as those for *A. mellifera*.

- Foraging activity: same as for *Apis*.

- Reproductive success (colony health) - Once it is known that the released female *M. rotundata* have successfully mated and started to provision cells (*i.e.*, either check tunnels to see if individual cells/eggs are present or look for sealed tunnels) assessments on increasing brood nest (*e.g.*, brood development) can begin. Check nest boxes on the first day after you know cell provisioning has commenced and then on a weekly or bi-weekly basis count and mark completed tunnels. Observation nests (grooved boards with clear acetate or glass covering the grooves) can be used to observe nest, cell, and brood development without disturbing the bees. At 15.6°C (60°F) eggs of *M. rotundata* take 15 days to hatch and then an additional 35 days for larvae to reach the prepupal stage. At 35°C (95°F) it takes 2-3 days for the eggs to hatch and 11 days for the larvae to reach the prepupal stage (Mader *et al.* 2010a). Therefore, if flowering of the study crop ends prior to either 14 days at 35°C or 50 days at 15.6°C, then the nest box needs to be removed from the study site and placed in a growth chamber that simulates the average temperatures experienced by the bees while they were in the enclosure. Once the prepupal stage has been reached, a segment of the styrofoam nest needs to be dismantled, cells per tunnel counted, cells weighed, and then dissected to determine the number of cells with prepupae and those that are provisioned but with no larvae present. If there are no larvae present (*i.e.*, these cells are called "pollen balls"), it indicates that larvae have died in the 1st or 2nd larval instar; and, which may be related to exposure to extreme temperatures (cold and hot) during that stage in development (Mader *et al.* 2010a). The remaining styrofoam nest sections can be dismantled, cells counted and then placed in storage at 2-5°C (35-40°F) at 50%RH until the following spring. At that time, the diapause can be broken and the number of emerged adults can be counted and compared to the total number of cells. This allows for determination of mortality in progeny (sub-lethal effects).

Semi-Field Studies – Social Non-Apis Bees

Bombus sp. will be used as the descriptive species in this section.

Bumblebee colonies are readily available from commercial sources[74]. A colony consisting of 50-300 workers and a queen can efficiently pollinate 1,000 m^2 to 3,000 m^2 (Morandin *et al.* 2001) of tomatoes, yet should also perform as a honey bee nucleus hive in a smaller enclosure (40m^2 to 60m^2). The 40 m^2 to 60 m^2 foraging area and considerations for supplying alternative forage (e.g. nectar or pollen) for honey bees is considered relevant for bumble bees. In addition,

[74] Worldwide, different bumblebee or alternative social non-*Apis* species are commercially reared for pollination purposes and, therefore, in most regions will not require import procedures (Mader *et al.* 2010b).

feeding bumble bee colonies can be done in a much more controlled way than *Apis*. When *Bombus* are commercially reared they are fed in the nest, and the same could be done for colonies used in a semi-field test. Colonies should be provided with the exact same amount of supplemental pollen and/or nectar, helping to minimize differences between treatments. Also, when changing food stores, one can remove the pollen or nectar that was not consumed and weigh it to determine just how much the colony ingested. A colony population of at least 100 workers and a queen should be used for semi-field studies, and exposure duration should be ten days followed by supplemental feeding. If the colony is movable then it may be appropriate to move it to a non-agricultural and pesticide free landscape to continue development out of the tunnel, rather than keep them inside the tunnel with artificial food.

When extracting bees for sampling, or mark and release, it is necessary to distinguish the queen (usually the largest bee) from the workers. Harm to the queen is likely to result in defensive behaviour on the part of the workers, and a rapid reduction in colony lifespan. Similarly, it may be desirable to distinguish between male bees and female workers. In general, male bumblebees have larger eyes, longer antennae, lack the enlarged hind legs with pollen baskets (corbiculae), and, depending on the species, may have a notable patch of yellow hair on the front of their face.

One to two *Bombus* sp. colonies of similar age and with at least 300 workers per colony should be moved to the semi-field study enclosure with entrances closed in the morning. Each colony should be placed on a concrete block with the entrance facing the morning sun. This should be done 2-3 days prior to the initiation of the study.

Key Outputs:

- Mortality in the crop: same as for *Apis*.

- Mortality at the hive: same as for *Apis*.

- Foraging activity: same as for *Apis*.

- Reproductive success (colony health). Prior to placing colonies in the semi-field enclosure a close-up picture should be taken of the brood nest and food stores through the plastic inner cover at night when most of the bees are back in the nest. The picture should be labeled with date and time and assessed for presence of brood in all phases of development by marking the cells with a marker on the picture.

  In addition, a small tarp can be placed under the colony extending outwards from the entrance so that any dead adults or drone larvae discarded by the colony can be counted over time. The tarp should be cleaned of all discarded adults and drone larvae after each assessment. Endpoints such as discarded dead adults and drone larvae are indicators of colony condition.

Semi-Field Studies – Stingless Species

The stingless bees Meliponini consist of approximately 24 genera of bees with around 400 species (the number is not clear as many species still remain to be described). They are important social bees in the subtropics and tropics (Nogueira-Neto, 1997). Meliponini occur mainly in Neotropical America, Australia, Indonesia, Malaysia, India and Africa (Proní, 2000). These bees are and have been important cultural components of many communities in the tropics and they are managed for their pollination services and honey production.

Stingless bees have varied nesting sites from aerial parts of trees to underground. They differ from *Apis* spp. in that their combs/cells are arranged horizontally and are mass provisioned by the nurse bees with nectar, hypopharyngeal gland secretions and pollen before the queen lays the egg after which the cell is closed. Full development through to the adult bee takes place within these cells without any further input by the nurse bees, hence each cell is representative of the conditions that existed during the construction and provisioning of the cells. A newly emerged bee destroys its cell immediately. Honey and pollen stocks are usually stored at the periphery of the nest with the brood in the middle of the colony. However, the arrangement of the brood and storage pots vary between species and for many species, these details remain unknown. It is believed that the adult workers have a similar life span to those of *Apis mellifera*, that is, they live 30 to 40 days.

Meliponini range in length from 1.8 to 13.8 mm (Michener, 2007) and, because of this, the choice of the species is important for risk assessment tests. One of the easier species to manage and rear in a lab is *Melipona scutellaris* (Uruçu ??? year of publication). In the past few years, *Melipona scutellaris* have been tested in glasshouses on tomato plants. In tropical areas some species such as *Trigona carbonaria* live and/or are managed in semi-domesticated situations.

Individual bees or the inner colony are easily accessed for testing. Individual bees can be chilled for several minutes in a freezer to slow their movement for ease of handling (the entire hive box should not be chilled). Heard (1999) and others have developed various hive box systems that can be used to manage these bees.

As regards to size of semi-field study, it is proposed that the approach used for the honey bee is adopted for the stingless non-Apis species.

Key Outputs: Details are similar to *Bombus* above.

Interpretation of Effects

As stated at the outset of this chapter, the interpretation of effects (i.e., a statistically significant difference from the control) is linked to the protection goals and, in particular, whether the results indicate that protection goals are likely to be met or not.

<u>If</u> the protection goal is pollination activity and/or function, then the semi-field study with measurements of foraging activity is capable of determining whether pollination activity is related to treatment. If there is an adverse effect on foraging activity in the semi-field study, then further information is required to determine whether the effects are realized at the field level. It was the view of the Workshop Participants that this would be best addressed via a field study. Alternatively, other information (such as …..), as well as consideration of risk mitigation may be elements of consideration in determining how to proceed.

<u>If</u> the protection goal is honey production, then the results from a semi-field study can be interpreted as follows:

- If effects are clearly *not seen* on any parameters then it can be inferred that there will be no impact on honey production at the field scale when full-sized colonies are exposed. This is assuming that long-term effects from short-term exposure were not an issue.

- If effects *are seen* or observed, *e.g.*, mortality or reduction in foraging or behaviour, then it may not immediately be assumed that honey production will be adversely impacted at the full-field scale. Since the semi-field test is potentially a worst case exposure scenario, the assessor needs to determine whether similar or any effect would be realized at the full-field level and hence whether honey production could be impacted.

If the protection goal is maintenance of biodiversity in terms of the ecosystem service of pollination by other non-*Apis,* then no negative impact on populations is the protective goal. Semi-field studies showing statistically significant effects that are expected to result in high levels of mortality should be considered for more refined field studies.

Assessment of the Variability and Uncertainty in an *Apis* Semi-field Study

As with any experimental testing, there are sources of variability and uncertainties associated with the studies. Confining organisms to a restricted study environment can confound efforts aimed at reflecting more environmentally realistic conditions. In the following section some of the sources of variability and uncertainty are discussed. To the extent that researchers can recognize and limit these potential confounding effects will likely improve the data generated from semi-field studies and improve their utility in regulatory decision making.

Parameter	Discussion of uncertainty
Enclosed population of bees	Under natural conditions, bees are free flying; enclosing them introduces a stressor that could lead to uncertainty in interpreting the results from a semi-field study. Enclosing bees in a semi-field setting causes two main issues, which may raise uncertainty when interpreting the results – (i) affects to behaviour and (ii) availability of food and therefore, and foraging activity. Food availability and foraging issues can be addressed through design considerations to ensuring is sufficient food availability. This can be achieved by balancing the size of the colony with the size of the enclosed crop. Details regarding possible size colony and area of crop combinations are discussed above.

Parameter	Discussion of uncertainty
	Providing a study designed to ensure that ample food is readily available and that there are comparable controls, should account for this potential confounding variable. Enclosing the bees could translate into behavioural affects, which could reduce exposure. For example, some bees will try to forage outside and as a result remain on the tent/cage wall rather than in the treated crop. It is not known what proportion of bees will exhibit this behavior. If the compound does not exhibit repellency effects on bees, it is thought that the same proportion of bees will potentially exhibit this characteristic in the controls as during the study. As there will be a proportion of bees that will not be exposed then this could potentially *underestimate* the risk. However, it is also not known what proportions of bees in the field are not exposed to the pesticide, *i.e.*, the proportion that will forage elsewhere. Providing that the population size is measured as a parameter, significant difference in comparison to controls indicate whether it is treatment related or not. It is considered that on the one hand exposure is confined and controlled; however, there will be a proportion of bees that try to forage elsewhere. Overall, participants of the Workshop believe that this parameter is likely to over-estimate potential risk, *i.e.*, it will be worst case.
Size of colony	The colony of bees that is used in semi-field studies is small compared with those used in the field; and the way that a small colony reacts is different than full-size colonies. Extrapolating effects related to mortality and sub-lethal behavior from a small colony to a standard colony is uncertain and should be approached with caution. Due to this uncertainty, if any effects are noted then further studies should be considered.
Measure of mortality	Due to the confined nature of the study it is likely that a semi-field study will yield a relatively accurate assessment of mortality. This is in contrast to the field where detecting an accurate level of mortality with in the crop is more difficult.
Density of bees in the treated crop	It is likely that the density of bees will be higher in a semi-field study compared to the field study. Due to the potential higher density of bees in a semi-field study compared to the field situation where alternative sources of food will be available, it is considered that bees are likely to have a higher level of exposure in a semi-field study, and therefore potentially over-estimate any effect.
Representativeness of the study site, agricultural practices	It is unlikely that there will be a study to represent every crop and geographical and agricultural combination being considered in the specific regulatory context. Hence, there will be uncertainty regarding the representativeness of the selected

Parameter	Discussion of uncertainty
and conditions	study site in comparison with possible combinations under regulatory consideration. Ideally the study site, in terms of weather, flower availability and forage should be designed to ensure that the bees are exposed. Uncertainty regarding the representativeness of the crop will be reduced if a surrogate is chosen that ensures that bees are suitably exposed. Addressing uncertainty based on agricultural and geographical variability is more problematic.
Residues in pollen and nectar	For pollen and nectar residue sampled from the plants, there is no reason to believe that these should vary any more or less than what would occur under field conditions, with the exception of no or limited exposure to rain (wash-off), wind or dew. Typically, semi-field studies have some latitude to make applications during periods of good weather. If poor weather is anticipated, then applications may be delayed several days provided the colonies are not already in the enclosure. However, semi-field studies are intended to reflect real world conditions, and if it rains, then such studies can still provide useful information. Typically, residue studies are conducted on the treated plants and in pollen/nectar to ensure that some level of exposure is achieved and the results are expressed relative to these residues.
Collected nectar, pollen pellets, bee bread and dead bees	Regarding nectar, there may be a high turnover rate in a semi-field study and therefore there may be difficulties in extrapolating this information to the field situation. Pollen and associated residues should be representative of what is likely to occur in the field and therefore the uncertainty associated with this parameter is low. Bee bread (i.e. fermented pollen/honey mixture that is stored in the comb) in a semi-field study is difficult to collect and the study has to be managed to ensure that this occurs. There is, therefore, some uncertainty regarding this parameter compared to what would happen in the field. Uncertainty exists if the study is extrapolated to other crops, for example if one species produces pollen and nectar whereas another species only produces pollen.
Assessment of the brood	This is only possible via OECD 75 and associated procedures.
Overall	Due to the confined nature there is high confidence that exposure will occur compared to a full-field study. It is also likely that any adverse behavioral affects will be seen. Therefore, if either increased mortality compared to the control or behavioral effects are not observed then it is considered highly likely that these will not occur in the field. Uncertainty exists regarding the potential effects on brood development; however, it is considered that this will lead to potential overestimation of the risk. Due to the duration of the exposure in the semi-field

Parameter	Discussion of uncertainty
	study, determine long-term effects requires special consideration. (see 1.6.1 for further details).

Design of a Field Study

When would a field study be appropriate?

Field trials may be carried out if an acceptable risk is not estimated by either lower tier tests or the proposed risk mitigation is undesirable. Questions to be answered from a field test should be based on the results of lower-tier studies, whether laboratory or semi-field. -For example, if behavioral effects are observed in a semi-field study, it may be desirable to see if these are observed under more realistic field conditions. It may also be more appropriate to conduct a field study where a semi-field study is not considered to be appropriate (*i.e.*, it is not necessary to always follow the tiered approach). For example, it may be relevant when there is the likelihood of long-term effects following short-term exposure. As with any test, involving animals, the need for and intent of the study should be clearly articulated. This is particularly true for field pollinator studies given the number of variables that must be managed, and the considerable resources they require both on the part of the regulated community to conduct the study as well as the regulatory authority tasked with reviewing the study.

Outline of a Field Study for *Apis* and Non-*Apis* Species

Design of a Field Study for *Apis mellifera*

Field trials can be used to address a range of exposure scenarios and effects. The results can be used by the risk assessor to determine whether significant uncertainties have been sufficiently addressed and if the protection goals may be met. However, there are various strengths and weaknesses of field studies that need to be considered before they are used in risk assessments intended for use in a regulatory context. Outlined below are the strengths and weaknesses of field studies. The strengths and weaknesses listed are relatively generic and relevant to tests employing either *Apis* or non-*Apis* bees.

Strengths of field studies
Provides a realistic exposure scenario of bees foraging on the crop, provided test plot size is sufficient
The realistic exposure scenario is likely to allow realistic behaviour of the bees
Can be designed to be consistent with good agricultural practice/grower standard practice.
Can be designed and used to assess longer-term exposure and effects (see below)
Ecologically (field level effects) and biologically (standard size colonies) more relevant than lower-tier studies
Can be relatively straightforward to conduct depending upon the aims of the study
Measurement of certain protection goals can only be, or are more accurately determined in field studies (*e.g.*, pollination deficit or honey production) assuming that lower tier studies have failed.

Weaknesses of field studies
Difficulty in finding appropriate sites, *i.e.*, there are practical issues in finding a site that is sufficiently isolated from other potentially attractive crops/pesticide treatments. Related to this is the potential for exposure via background level(s) of pesticides in forage areas.
Because field studies are open, controlling nutritional sources may be difficult as bees may not forage exclusively within the treated field
Expensive to establish treatment area of size suitable for indicating "worst case" exposure. Field studies are logistically complex and are expensive since so many factors have to be accounted for.
Potential difficulty related to background levels of pesticides in the foraging area
Difficult to use toxic standard which in turn potentially raises concerns regarding sensitivity of the test system.
Potential high level of variability including weather, mortality away from the hive, replication and interpretation of results

Study Design Considerations

*All types of application (*i.e.*, spray, systemic solid formulation/seed treatments/soil treatments applications)*

The study should use colonies with a minimum of 10,000-15,000 foraging bees. Colonies should consist of 10-12 frames and include 5-6 brood frames. If colonies are of a different size then they should be evenly distributed between treatments. According to EPPO 170 an area of 2,500 – 10,000 m^2 (0.25 - 1 ha) is recommended with a larger area proposed if the crop is not particularly attractive (*e.g.*, 0.25 ha for *Phacelia* and 1 ha for mustard and oilseed rape). EPPO 170 also recommends that there should be a minimum of 4 colonies per field. It may be appropriate or necessary depending upon the regulatory question being asked, to consider the use of larger field sizes as this may provide a greater degree of realism when compared to the eventual use of the product. If larger fields are used and depending upon the attractiveness of the crop, then more colonies may be required. It is important to determine, from scientific literature, the proper colony loading rates based on crop and size of field. In determining the size of individual fields consideration must be given to the total number of treatments (i.e., the treated crop) and replicates per treatment (i.e., colonies per treated field).

While replication of treated plots is ideal, it is appreciated that this is unlikely to be feasible. This issue is dealt with further in the STATS CHAPTER.

While it is potentially desirable to use a positive control in a semi-field study, it is discouraged in a full-field study. This recommendation is based on extensive discussion among the ICPBR and EPPO. A negative control, however, is always required.

Participants of the Workshop agrees that bees generally tend to forage on sources close to the colony, but that some bees will forage further afield and these individuals could bring additional residues into the colony. Consequently, in order to ensure adequate isolation from other (alternative) sources of pollen and nectar, the site should be located at least 2-3 km from alternative cultivated sources of pollen and nectar, including pollen and nectar from trees. As regards confirming exposure, the following measurements should be considered:

- Bees/m^2 – at least five bees per m^2 on *Phacelia* spp. or 2-3 bees per m^2 on oilseed rape and mustard (EPPO 170). These are potentially only relevant for these crops and EU conditions and should be used with caution in other regions. It should also be noted that these densities are related to the number of colonies and size of treated area.

- Pollen identification – It is recommended to have additional colonies with pollen traps fitted. Identification of pollen can be difficult and sometimes identification is only possible to family level (not at the genus, or species level).

If appropriate, there should be an assessment of the degree of flowering, i.e. the proportion of the crop actually in flower at any one time (*e.g.*, BBCH 60 onwards for oilseed rape (see [HYPERLINK "http://pub.jki.bund.de/index.php/BBCH/article/viewFile/470/420"] for further details). This is particularly relevant for crops such as melons. It may, under certain conditions, be possible to manage the crop to prolong flowering so that continual exposure could result.

For systemic chemistries, it is not possible to identify a suitable positive (reference) standard. In addition, similar to considerations with systemic chemistries under a semi-field design, exposure will occur over a longer time, therefore, the honey bees should be present during the whole flowering period of the plant. Acclimation to the pesticide will occur as soon as they are introduced in to the treatment area. However, a consideration of mortality due to moving the colony is still required. One potential way around this is to compare the mortality that occurs on the untreated crop to that in the treated crop. Nevertheless, the significance should be determined statistically (Ref Stats chapter).

Pre-application

For all application types. Pre-application considerations are similar to that for semi-field studies. Refer to these sections above.

Post-treatment assessments

*All types of application (*i.e., *spray, systemic solid formulation/seed treatments/soil treatments applications)*

Depending upon the regulatory question being asked, it may be necessary to assess behavioural effects in the field. Mortality, however, should always be determined. While this may be done via the use of dead bee traps, these may not always be appropriate in which case sheets or tarps outside the hive should be used.

A key issue with field studies is ensuring that sufficient exposure occurs. Study designed can minimize alternative forage, however it is inevitable that there will be some alternative sources present In order to determine whether exposure has occurred, there is a need to monitor the activity of bees within the treated crop. This can be done one of several ways.

- Measuring forage activity:

  See previous discussion on measuring foraging activity, under Section x.x.x. "Design of Semi-field Study for Apis bees" subsection "Post Treatment Measurements"
- Measuring flight activity:

  Aided through the use of marked bees
- Pollen identification outside the colony

- Measuring residues in pollen and nectar in bees and inside the colony

Closely related to this point is whether the exposure that has occurred will be representative of the wide-scale use of the pesticide.

Results

The following measurement endpoints and outputs are possible from a field study:

- Colony strength: ascertained through measurements of forage activity, flight activity and number of dead bees.
- Weight of the hive
- Pollen, honey and nectar stores: ascertained through measurement of percent comb coverage.
- Mortality at the hive: ascertained through measurements with dead bee traps or collecting sheets
- Mortality of drones and pupae: ascertained through visual inspection of frames
- Mortality in the crop: ascertained through collection sheets in the treatment site.
- Presence of the same queen
- Foraging activity in the crop: measured at the food source or at the hive entrance and can be counted automatically
- Returning foraging bees: can be counted automatically
- Behavioural abnormalities
- Residues in pollen, nectar, pollen pellets, as well as residue measurement in wax, bee bread and dead bees: measurements of exposure inform assessment of risk.

- Assessment of the brood: see EPPO 15; this measurement may also include an estimate of the number of adults, the area containing cells, eggs, larvae and capped cells)
- Disease and/or pest levels

It is important that the study is designed so that measurement endpoints are statistically valid, (see Chapter X).

Long-term Risk to Honey Bees from Short-term Exposure

If potential over winter effects is identified during the problem formulation step, then it is proposed that the field study is modified in order to examine measurement endpoints that will address this uncertainty. (Generally, field studies are more appropriate to assess the impact of over wintering than extended semi-field studies.)

If a field study is to be conducted to determine whether the use of a product has any adverse effects on overwintering survival, then it is proposed that in addition to the considerations discussed above, the following points are also considered:

Following the exposure phase the colonies (treatment and controls) should be re-located to a new location with limited to no agricultural crops and an abundance of natural vegetation. This is necessary to ensure that exposure to additional pesticides does not occur.

At the end of the winter period, it is proposed that the following assessment endpoints should be determined, however, the exact endpoints will depend upon the issues highlighted in the problem formulation.

- Condition of the colonies,
- Brood development,

- Brood assessment, including:
    - Strength of colonies
    - Presence of healthy egg-laying queen
    - Estimate of pollen and nectar storage areas
    - Estimate of areas containing eggs, larvae and capped cells
- Analysis for disease, (*e.g.*, *Nosema apis*, *Varroa destructor*, American foulbrood, bee viruses)
- Weight of the colonies
- Residue samples from the hive (*e.g.*, pollen, wax, honey, bees)

Interpretation of Effects

As for semi-field studies, the interpretation of effects is linked to the protection goals, identified above. It should be noted that while a full-field test is the highest tier of testing it is important that final determination of potential risk, and whether the use of the compound is consistent with protection goals should be based on the entire body of evidence across all tiers.

If the protection goal is pollination activity or pollination function, then the full-field study is capable of determining whether this is achieved via use of measurements on (i) foraging (which can include foraging for nectar and pollen), (ii) behaviour and, (iii) mortality. If no effect is observed on any of these parameters then the protection goal will be met. If effects are seen on any of these parameters then it is *unlikely* that the protection goal will be met. (It should be noted that none of these are directly related to pollination activity and therefore they are surrogate measures for the actual protection, i.e. in using foraging activity it is assumed that a decrease in foraging activity will result in a decrease in pollination *e.g.* fruit set.)

If the protection goal is honey production by the colony, then this study can provide useful information. For example, if there are clearly no effects then it can be inferred that there will be no impact on honey production. If statistically significant effects are observed over the course of the study, then it can be concluded that the protection goal of no adverse effects on colony productivity will not be met.

Design of a Field Study for Non-*Apis* Bees

Given the lack of investigation into a field level test for non-*Apis* species, it is assumed that all non-*Apis* bee testing will be in conjunction with field studies that are primarily designed for *Apis* bees.

Outlined below are draft protocols that could form the basis of field studies conducted to address specific regulatory questions.

Field Studies - Solitary Bees:

Megachile rotundata will be used as the descriptive species in this section. It is also important to note that *M. rotundata* and *Osmia* sp. have a much more restricted foraging range (approximately 300 m) than *A. mellifera* (2-3 km); therefore, it is much easier to ensure that their foraging will be restricted to the crop at the study sites.

Preparation of *M. rotundata* for these studies should be undertaken using the same maintenance and handling protocols described for *M. rotundata* in the semi-field study.

Key Outputs

Key outputs include mortality (in the crop and at the hive/nest) foraging activity, and reproductive success (as a measure of colony health). Assessment of these endpoints is similar to that for *Apis* tests (see above).

Field Studies - Social Non-Apis Species

Bombus sp. will be used as the descriptive species in this section. It also is important to note that *Bombus* sp. have a much more restricted foraging range (400-750 m) (Knight *et al*. 2005) than *A. mellifera* (2-3 km) therefore it is much easier to assure that their foraging will be restricted to the crop at the study sites.

Preparation of *Bombus* sp. for these studies should be undertaken using the same maintenance and handling protocols described for this species group in the semi-field study.

Key Outputs

Key outputs include mortality (in the crop and at the hive) foraging activity, and reproductive success (as a measure of colony health). Assessment of these endpoints is similar to that for *Apis* tests (see above).

Field Studies –Stingless Species

Stingless bees (Meliponinae) have a social life similar to the honey bees albeit in much smaller colonies. There is an increasing body of literature (Heard 1999, Amano 2004) showing the value of stingless bees in pollination of crops in tropical and temperate countries. The stingless bees are native to tropical and subtropical areas where they occur, with more than 400 species having been recorded from these regions. The ease of handling these species (small colony sizes, and hesitance to sting) makes them ideal candidates for pollination in glasshouse conditions. In addition, since they are active all year, they pollinate crops that honey bees are unable to (Amano 2004). However, in terms of their use for pesticide tests, there is very little information and thus the information below should be taken as a guide with allowance for improvement. It is expected that this guidance document will create interests among the practitioners to develop and validate methods and create a forum for revisions in the future, if required.

Hives

Hives for stingless bees are box shaped (commercial units) but smaller compared to those of honey bees. They do not have frames rather they are hollow, containing the whole colony component. Opening the hive therefore should be done gently to avoid damaging/destroying the nest structure. Honey and pollen are stored in pots made of beeswax. The pots are typically arranged around a central set of horizontal brood combs. When the young worker bees emerge from their cells, they tend to remain inside the hive, performing different jobs. As workers age, they become guards or foragers. Unlike the larvae of honey bees, Meliponine larvae are not fed directly. The pollen and nectar are placed in a cell, an egg is laid, and the cell is sealed until the adult bee emerges after pupation (*i.e.*, mass provisioning). At any one time, hives can contain 300-80,000 workers, depending on species.

Stingless bee colonies can be purchased from beekeepers that specialize in stingless bee production and management. Stingless bees that are currently commercially available in tropical countries include (but are not limited to), *Melipona beecheii, M. quadrifasciata, Trigona carbonaria, Tetragonula fuscobalteata, Scaptotorigona bipunctata, Tetragonisca angustula, Meliponula ferrugenea, Hypotrigona gribodoi,* and *Meliponula bocandei*. See Non-*Apis* chapter (Chapter ##) for details on which species are appropriate for specific countries.

Care should be taken to acquire strong colonies with sufficient workers, each with about 10,000 healthy foragers, however this will depend upon the species used. Up to eight colonies per ha may be used. Stingless bee hives can be placed at strategic positions similar to operating with honey bees (*e.g.*, either in the middle or edge of the field); and, hives should be sheltered with a wooden cover placed on top of the hive to avoid direct rainfall on the hive.

Stingless bees have a wide foraging range, foraging up to 2.1 km (Kuhn-Neto *et al*. 2009), but on average will restrict their activity to within 1 km of the colony. The isolation distance from other forage sources recommended for honey bees (2-3 km) can thus be used.

The number of individuals per hive and per unit area recommended for honey bees can also be applied for the stingless bees. However, noting that there have been no field tests of this kind done for stingless bees, there is research need to validate the protocol.

Treatment Application, Sampling, Data Analysis and Interpretation

Same as for *Apis*

Key Outputs:

The end points for the stingless bees in the field tests are similar to the honey bees, and include:

- Colony strength
- Hive weight
- Pollen, honey and nectar stores
- Mortality at the hive (via the use of dead bee traps or collecting sheets)

- Mortality of drones and pupae
- Mortality in the crop
- Presence of the same queen
- Foraging activity in the crop
- Returning foraging bees
- Behavior
- Residues in pollen, nectar, pollen pellets, wax, bee bread and dead bees (i.e., measures of exposure)
- Assessment of the brood (including an estimate of adults, the area containing cells, eggs, larvae and capped cells)

Assessment of the Uncertainty in a Field Study

Unlike lower-tier studies with insect pollinators, environmental conditions are far less easy to control in full field studies. Additionally, although sources of variability and uncertainty may exist, there may be fewer options available for researchers to address these issues under full field conditions. While many of the options available for semi-field studies may apply to full field studies, the logistics of stratifying designs and increasing the number of replicates become logistically difficult to implement.

Parameter	**Discussion of uncertainty**
Exposure	Uncertainty of exposure should be minimized by proper location of the site in relation to other foraging sites; ensuring that the target crop is maximally attractive to bees. Determination of exposure can be made through measurements (as discussed above for *Apis* species). As with *Apis* tests, it is essential that there is information on the degree of exposure in determining the usefulness of the study.
Location of site(s)	The location should be relevant for the crop and environmental conditions (climatic, botanical and edaphic) both when and where the study is conducted. The likely reality is that tests cannot be conducted for all crop/formulation/geographic combinations and so there may be uncertainty when extrapolating the results. The uncertainty could over and under-estimate the risk

Parameter	Discussion of uncertainty
	depending upon the actual study in question and the uses/situations to which it is being extrapolated to.
Difference between the treatment areas and the controls	It is possible that the control and the treatment areas may differ both in terms of climate and edaphic conditions. Any differences in the testing environment (i.e.., vegetative surroundings, climatic, or edaphic) should be minimalized.
Extrapolation between different varieties and sub-species of bee	Only one bee species or subspecies will be tested in one study, Uncertainty will exist when extrapolating inter-species, but may also exist when extrapolating intra-species. For example, while there is information indicating that effects on *Apis mellifera mellifera* and *Apis mellifera carnica* are minimal, *i.e.*, they are of relatively similar sensitivities, the differences in sensitivity between *Apis mellifera scutellata* and subspecies of European honey bee is unknown, and *Apis mellifera scutellata* may be more or less sensitive than the European honey bee.
Mortality away from the hive	Measurement of mortality away from the hive will be difficult and therefore there will be much uncertainty in this parameter. It would not be reasonable to expect that any measurement endpoint can be thoroughly documented and in most case, the best the study can do is detect relative differences between control and treated colonies. Dead bee traps are likely prone to the same biases in control and treated fields. It might be argued that predatory/scavenger insects may be reduced in treated fields relative to untreated fields and that there is a lower likelihood that dead bees may be removed from traps whereas in control fields greater scavenging may occur making it appear as though mortality was lower in the untreated field. This underscores the need to calibrate dead bee traps to determine the efficiency of recovery. This parameter will potentially underestimate any level of mortality. However, other measurements, *e.g.*, colony health (strength and weight) will provide an indirect measure of mortality (*i.e.*, if much mortality occurs away from the colony then it is likely that the overall hive health/colony development etc will be adverse affected.)
Overall	A field study is an assessment of the potential effects on the colonies under realistic climatic, botanical and edaphic conditions. There are uncertainties regarding the degree to which bees are exposed although the resulting exposure is likely to represent more normal conditions than those in a semi-field studies. There are uncertainties regarding the sensitivity of the bees tested as well as extrapolating the study to other sites, situations and crops, however, these should be assessed on a case-by-case basis.

Role of Monitoring and Incident Reporting

Some countries have monitoring schemes aimed at providing information on regulatory decisions. These schemes provide feedback on the quality and accuracy of the the regulatory decisions, and therefore by association elements of that decision such as measurement endpoints, assessment endpoints, up through protection goals). In addition, some regulatory authorities require monitoring of bee colonies as a condition of registration where the likelihood of potential risks could not be reduced sufficiently.

Monitoring schemes, for example the UK Wildlife Incident Investigation Scheme (WIIS) rely on incidents being reported to a central organisation. This scheme has provided much information on incidents resulting from both the correct use as well as accidental incorrect or misuse as well as abuse. These data, along with usage data, have been useful to determine the appropriateness of various regulatory restrictions as well as providing information on the appropriateness of the regulatory trigger values (see Aldridge and Hart, 1993, and Mineau *et al*., year). In North America (under the USEPA system) pesticide registrants are required to report incidents when they become aware of them. Other stakeholders may also report incidents to the USEPA.

These schemes do, however, have limitations in that they are rely on the public to both find an incident, but also to report it. This can potentially lead to under-reporting if the beekeepers fears retrabution , or the citizen is unaware of the process of reporting. The conditions of commercial agriculture verses that of native wildlife predispose reporting to be bias toward *Apis mellifera,* consequently, incidents involving non-*Apis* bee species may be under recorded. Nontheless, monitoring schemes are a useful tool to the regulator to better understand the use and effects of pesticide compounds. Cost-effective reporting schemes need to be developed that provide incentives to applicators to help increase reporting of experiences from the field. This is critical for improving risk assessment and mitigation.

Summary/Conclusion

TO BE DEVELOPED? This should be developed after the document content is finalized

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Chapter 10 Overview of a Proposed Ecological Risk Assessment Process for Honey bees (*Apis mellifera*) and Non-*Apis* Bees

Ecological risk assessments are intended to evaluate the likelihood that adverse ecological effects may occur as a result of exposure to one or more stressors (USEPA 1992[75]). Typically, at the first tiers, risks are evaluated for individual taxonomic groups (*e.g.*, freshwater fish, upland game birds or terrestrial plants) using surrogate species. At higher levels of refinement, risks to individual taxa may be further integrated to determine whether there are effects to the community; however, risk assessments are typically conducted at the taxon level (USEPA 2004). The intent of this chapter is to describe a proposed method for estimating risk to honey bees (*Apis mellifera*) and non-*Apis* bees from pesticides that are applied via sprays (acting on contact) and via seed/soil treatments and tree trunk injections (acting systemically).

In general, a pesticide risk assessment process is used for evaluating new compounds or new products entering the market or those compounds undergoing re-evaluation, as in the 10-year process of re-evaluation in the EU or in the North America where chemicals are re-evaluated every 15 years. As with risk assessments for other taxa, the proposed risk assessment method described in this document makes use of surrogate species. The ecological risk assessment process described consists of a series of steps or phases which are intended to be iterative where information gathered at each step is evaluated against the protection goals. The risk assessment process consists of a problem formulation (Phase 1), analysis (Phase 2) and risk characterization (Phase 3). This generic process is depicted in **Figure 1**. In Phase 1, *problem formulation*, measurement endpoints are selected in relation to protection goals and corresponding assessment endpoints, a conceptual model is prepared and an analysis plan is developed. Based on the conceptual model and its associated risk hypothesis, the analysis plan articulates how the risk hypothesis will be tested. In Phase 2, *analysis*, available measures of exposure and

[75] U.S. Environmental Protection Agency. 1992. Framework for ecological risk assessment. Washington, DC: Risk Assessment Forum, U. S. Environmental Protection Agency. EPA/630/R-92/001.

measures of effect are evaluated. Through environmental fate data, the movement of a stressor (*i.e.*, the pesticide and relevant transformation and breakdown products) in the environment is characterized; this is frequently termed the exposure characterization or exposure profile. Similarly, the potential acute and chronic effects of a chemical are characterized in what is frequently termed the stressor-response profile. Additionally, the proposed and/or existing uses of a compound are characterized and based on these uses and the environmental fate of the compound, predicted/estimated environmental concentrations (PEC or EEC) are derived.

Once effects and exposure are characterized, the risk assessment proceeds to Phase 3, *risk characterization*. Typically, the risk characterization consists of two steps, *i.e.*, risk estimation and risk discussion (evaluation). In the risk estimation step, the measures of exposure (*e.g.*, EECs or PECs) and measures of effect are integrated to develop risk estimates. These risk estimates may be based on point estimates of exposure and a point estimate of effect, *e.g.*, for tier 1, exposure is based on application parameters assumed to result in the highest exposure for a particular use, and point estimates of effect, *e.g.* the acute median lethal dose to 50% of the species tested (LD_{50}). If initial values for potential exposure and effects result in risk estimates that exceed regulatory triggers, then these point estimates can be refined through higher tier testing with regard to both potential exposure and/or potential effects. Possible refinements in exposure estimates are discussed in Chapter 6 while possible refinements in effects are discussed in Chapter 7 (laboratory studies) and Chapter 8 (semi-field/full field studies). As ecological risk assessment methodologies evolve, refined estimates could be based on distribution-based estimates of either exposure (*e.g.* residue concentrations in pollen from field monitoring studies based on application rate reflecting the worst case for a particular use), or effects (*e.g.*, species sensitivity distribution using LD_{50} values for non-*Apis* species). Regardless of whether point estimates or distribution-based estimates are used, the integration of exposure and effects data is typically expressed as a ratio (quotient) of exposure and effect estimates and it is this ratio which is considered to be the "risk estimate". If point estimates of exposure and effects are used as inputs, the risk quotient is a deterministic point estimate of risk. If the exposure and/or effects inputs are probability distributions of possible values, the risk estimate is itself a "joint" probability distribution and represents a probabilistic estimate. Deterministic estimates of risk, based on point estimates of exposure and effects, do not typically provide information on the magnitude and likelihood of adverse effects. (This uncertainty is in most cases accounted for with the use of assessment factors.) In refining the risk assessment on the basis of distribution-based estimates of either or both exposure and effects, probability distributions and particularly joint-probability distributions allow the estimation of both the likelihood (probability) and magnitude of an adverse effect (*e.g.*, estimates of a 40% chance that 60% of the species will be affected). The decision to move from point-estimate based approaches to distribution-based approaches that may also be spatially and temporally specific is predicated on the risk manager's need for additional information to support their decision and on the need[76] and availability of data to support such approaches.

The second part of *risk characterization* is risk evaluation where quantitative estimates of risk are, when necessary, further described using qualitative data. Multiple lines of evidence are used to more fully describe what is known about potential adverse effects resulting from the use of a pesticide. Risk evaluations include additional discussion about the variability associated with the measured endpoints along with associated uncertainties, *i.e.*, attempts to characterize what is not known. When necessary or possible, the intended effects of relevant mitigation measures may also be discussed. Any incident data, *i.e.*, adverse effects reported involving the actual use of the compound in the field, are also discussed to further characterize potential effects.

Although the risk assessment process is depicted as three distinct phases, each phase is intended to be iterative. As more information (data) becomes available, the outcome of the process should evolve to accommodate to the data. The risk assessment process is therefore intended to take advantage of multiple lines of evidence and the problem formulation with its conceptual model and risk hypothesis may be refined as more

[76] Species sensitivity distribution are an option to refine the evaluation of effects for risk assessment performed for a group of organisms and not a the level of a species *e.g.* the honey bee.

information becomes available. A critical component to this iterative process is clear communication between the risk assessor and the risk manager to insure that protection goals are adequately accounted for and that the relevant mitigation measures on risk estimates may be implemented and potentially evaluated within the risk assessment.

Consistent with the iterative nature of the risk assessment process, regulatory authorities typically rely on a tiered process for conducting ecological risk assessments; the preliminary, or screening-level (Tier 1) assessments are intended to screen substances for which a potential risk cannot be excluded. Higher tiers attempt to refine risk estimates to (*i*) identify whether a potential risk will likely be encountered under more realistic assessment conditions, *i.e*, using less conservative assumptions regarding potential exposure and effects, (*ii*) determine the conditions under which potential risks may occur; and (*iii*) identify spatially- and temporally-specific risks. The tiered risk assessment process identifies those chemicals for which a higher level of resources should be devoted to support more refined and detailed assessments. It should be noted though that while probabilistic tools can be used to refine estimates of exposure and effects, and to quantify spatially and temporally-specific risks, they are not typically applicable to determining the conditions of occurrence for risk.

Decision criteria are used within a tiered framework as a basis for discriminating potential risk(s) among substances. Screening-level risk assessments may have predetermined decision criteria to answer whether potential risks exist, as for example in the EU where decision-making criteria are defined for all groups of organisms (EC, 2001). Conversley, higher tier risk assessments may not have predetermined and/or uniformly defined decision criteria since the management decision may change from yes/no to questions regarding "what, where, and how great is the risk", as for example in the US (USEPA 1998[77]) and may also be associated with restrictions/conditions intended to limit risk (which is the case in both the EU and US).

[77] U.S. Environmental Protection Agency. 1998. Guidelines for Ecological Risk Assessment. . Washington, DC: Risk Assessment Forum, U. S. Environmental Protection Agency. EPA/630/R-95/002F

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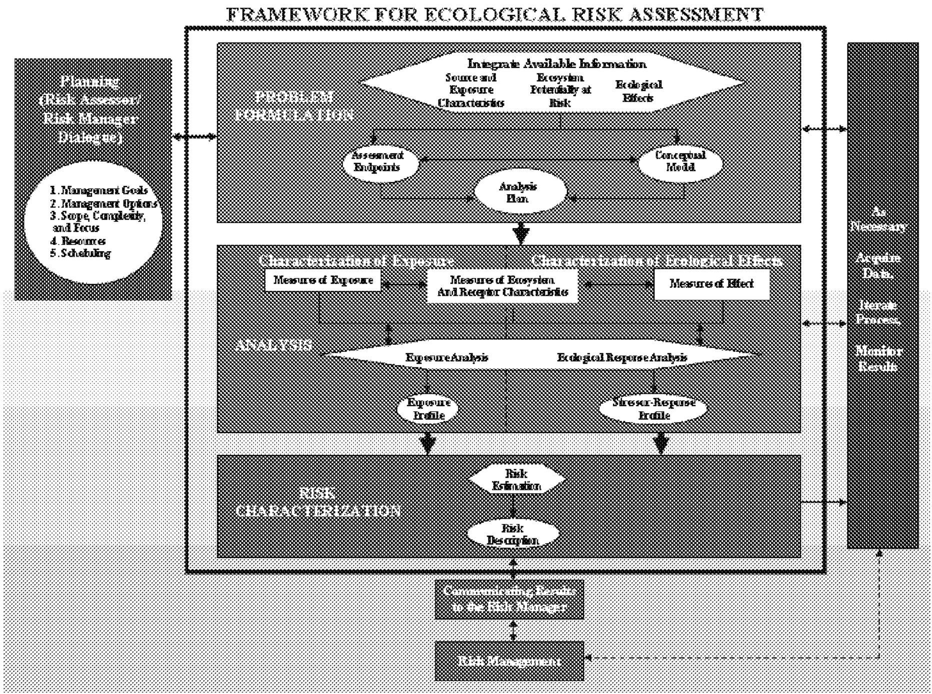

Figure [SEQ Figure * ARABIC]. Diagram of Ecological Risk Assessment Process employed by US EPA

In the following sections, the risk assessment process for honey bees and non-*Apis* bees is articulated. Consistent with the tiered process discussed in the preceding sections, the following sections propose risk assessment flowcharts discussed during the workshop and are intended to illustrate the different steps mentioned above. Each step of these risk assessment processes are then discussed in greater detail, starting with screening-level assessments (Tier 1) and proposed refinements that incorporate additional data on potential exposure and effects to both *Apis* and non-*Apis* bees. The proposed process is delineated for pesticides that are applied foliarly and act on contact with or ingestion by insects. A different risk assessment process is articulated for pesticides that are applied to soil or as a seed treatment. For soil and seed treatments that are systemic, the chemical is taken up by the plant and distributed either through xylem (*i.e.*, translocation through the plant in the direction of xylem flow (acropetal) or through plant phloem (*i.e.*, translocation through the plant in the direction of phloem stream (basipetal and acropetal). The route of exposure to systemic compounds applied as soil, seed or tree trunk injections is primarily through ingestion of residues in pollen and/or nectar.

Definition of protection goals, assessment and measurement endpoints, and trigger values for transitioning to higher levels of refinement

As previously discussed, the initial phase of a risk assessment process is problem formulation. The problem formulation articulates the intent of the risk assessment and is predicated on particular protection goals for which the regulatory authority is responsible. In order to build a proposed risk assessment process for pollinators, the participants of the Workshop identified plausible, surrogate protection goals; these included:

(i) protection of pollination services provided by *Apis* and non-*Apis* species'
(ii) protection of honey production and other hive products; and,
(iii) protection of pollinator biodiversity,

In order to structure an assessment that allows addressing risk management concerns, *i.e.*, realize protection goals, it is important to define assessment endpoints. Assessment endpoints are intended to be explicit expressions of the actual environmental value that is to be protected and are operationally defined by an ecological entity and its attributes (USEPA 1998). For assessing potential risks to *Apis* and non-*Apis* bees the ecological entities would be the organisms themselves (*e.g.*, larval and adult honey bees and bumble bees) and potential attributes would consist of survival, development and reproduction. The ability of assessment endpoints to support risk management decisions depends on the extent to which they target susceptible ecological entities and measurable ecosystem characteristics (USEPA 1998). Protection of the growth, reproduction and survival at the colony/population level of these species will conserve pollination services; biodiversity contributed by pollinators, and availability of hive products (*e.g.*, honey production). The conventional assessment endpoints of survival, development and reproduction can be articulated for Apis and non-Apis bees to include colony size and survival (for honey bees) and population size and survival for (non-*Apis* bees).

Assessment endpoints are further defined by measurement endpoints. Measurement endpoints are attributes that are examined at the study level which, either individually or taken together are indicative of an assessment endpoint. In initial [screening level] laboratory studies it is practical to measure endpoints such as individual survival, toxicity on and developmental effects to larvae (brood), and behavioural effects (*e.g.,* effects that become manifest in adults due to exposure as larvae). These measurement endpoints are relevant because if severely impacted, they can result in effects at the colony/population level and can be indicative of the ability of a colony to grow, reproduce, or survive. In higher tier tests, it may be possible to directly measure effects on colony/population size and viability. (However, as noted in previous chapters, further research is required to ascertain which [sublethal] effects and at what level of perturbation is indicative of a colony-level, or population-level effect.) The linkage between protection goals, assessment endpoints and possible measurement endpoints are presented in **Table 1**.

Table X. Linkage of protection goals, assessment endpoints, and measurement endpoints for social bees (including *Apis*) and solitary (non-*Apis*) bees. Initials (L) and (F) designate endpoints most applicable to laboratory studies and field studies respectively.

Protection goal	Assessment endpoints	Measurement endpoints Population Level or higher	Measurement endpoints Individual Level
Pollination services	Population size and persistence on the crop/in the boundaries	Social bees: Colony survival (F), colony strength (F) Solitary bees: Population size (F) and persistence (F) over time	Social bees: Individual survival (L, F), fecundity (F), brood success (L, F), behavior (L, F) Solitary bees: Individual survival (L, F), reproduction (F), behavior (L, F)

Hive products (honey, *etc.*)	Production of hive products	Production of hive products (F)	Individual survival (L, F), brood success (L, F), behavior (L, F)
Pollinator biodiversity	Species richness and abundance on the crop/in the boundaries	colony survival (F), colony strength (F), brood success (F), behavior (F) Species richness and abundance (F)	individual survival (L, F), brood success (L, F), behavior (L, F)

Screening-Level Risk Assessments (Tier 1)

As noted above, ecological risk assessments typically follow a tiered process (depicted in **Figure 1**). Substances move through lower tiers to higher tiers when the information indicates potential risk cannot be excluded. The first tier of that process is the screening-level assessment, which is intended to effectively and rapidly:

- exclude substances of low risk concern from entering into resource intensive higher tier risk assessment; and,
- identify substances for which a potential risk to bees cannot be excluded and for which a higher tier risk assessment is needed.

The screening-level assessment should allow for the most efficient allocation of resources and minimize the number of substances forwarded for higher tier evaluation while still identifying those of potential risk to bees. An efficient screening step in the risk assessment is essential as it optimizes the success in achieving protection goals based on appropriate risk assessments. At a screening-level, the intent is then to use an appropriately sensitive species that is suitable to ensure that protection goals will be met. In this context, in designing the risk assessment process, participants proposed the *A. mellifera* as a reasonable surrogate for both *Apis* and non-*Apis* bees at a screening level for evaluating acute toxicity to adults. The reasons for this are:

- the biology and availability of *Apis-miliffera* readily lends itself to testing and analysis;

- tiered toxicity test guidelines are widely available for this species; and,
- conducting and interpreting the results of these tests does not require specialized backgrounds and/or conditions.

As illustrated in the flow chart depicted in **Figure 1**, the screening step most often relies on the calculation of risk estimates, termed Risk Quotients (RQ), Hazard Quotient (HQ) or Toxicity Exposure Ratios (TER). These risk estimates are compared to numerical regulatory decision criteria, termed a “Level of Concern” (LOC) or “trigger criterion”. A trigger value typically accounts for uncertainties related to intra- and inter-species variation in sensitivity, extrapolation of short-term toxicity to long-term effects, and extrapolation of laboratory results to the field.

Depending upon the type of risk estimate used (RQ or TER), if the estimate is above, or below the LOC than a determination of minimal risk is presumed, or whether additional refinements are necessary. For example, if screening-level risk estimate results in a TER (where the effects estimate is divided by the exposure estimate) that exceeds the trigger value, then minimal risk is presumed (i.e., if TER > X = minimal risk is presumed); conversely, if the TER is does not exceed the trigger value, then minimal risk cannot be presumed, and a higher tier risk assessment may be needed. The RQ, is the reciprocal of the TER in that the exposure estimate is divided by the effecs estimate; therefore, the RQ value is interpreted opposite to the way in which the TER is interpreted, *i.e.*, if the RQ exceeds a trigger value, then minimal risk is not presumed and a higher tiered risk assessment may be needed (i.e., if RQ < X = minimal risk is presumed). If the RQ is greater than the trigger value (or LOC), then minimal risk is not presumed.

The terminology of risk assessment can be confusing due to the differences amongst regulatory authorities. Many parts of the processes outlined in this document make reference to the European EPPO methodology, and the testing methods for non-target terrestrial arthropods thereof. **Table X** presents the different risk expressions.

Table X. Risk estimates and their component parts used by regulatory authorities.

Ecological Risk Estimate	Effects Component	Exposure Component	Comment	Where/How it is Used
Hazard Quotient (HQ): Effects/Exposure	LD_{50} measured as ug/bee	Dermal exposure concentration or oral dosing concentration as g/ha	Numerator and denominator are expressed in dissimilar measurement units	Used in European assessments Used in Tier 1 analysis
Risk Quotient (RQ): Exposure/Effects	LD_{50} measured as ug/bee	Contact exposure concentration, or oral dose concentration	Numerator and denominator are expressed in same measurement units	Used in North American assessments Used in Tier 1 analysis
	No Observed Adverse Effect Level (NOAEL) measured as ug/bee	Oral feeding concentration (solution) or dietary intake (pollen or nectar)	Numerator and denominator are expressed in same measurement units	Used in North American assessments Can be used in Tier 1, and Tier 2, analysis
Toxicity Exposure Ratio (TER): Exposure/Effects	No Observed Adverse Effect Level (NOAEL) measured as ug/bee	Oral feeding concentration (solution) or dietary intake (pollen or nectar)	Numerator and denominator are expressed in same measurement units	Used in European assessments Used in Tier 1 analysis (for larvae) and Tier 2 analysis

Note that in Tier 3 analysis, where a field study is performed, neither an HQ or RQ nor a TER is calculated. Rather, effects are characterized, statistically significant or not, in context of actual exposure conditions and in context of whole hive biology.

Risk Assessment Flowcharts

The following section illustrates the proposed risk assessment process identified by the participants of the 2011 SETAC Workshop on Pesticide Risk Assessment for Pollinators. The decision process is described, and depicted in flowcharts to better highlight the progression of events through the tiers. Risk assessment starts with a preliminary verification that a risk assessment is warranted by first describing routes of exposure that are considered likely and will trigger further evaluation. This leads to screening steps intended to exclude situations where the potential for adverse effects is considered low and with a sufficient margin of safety to conclude no further analysis is necessary. The process then focuses on uses for which further characterization of the risks is necessary and guides the assessor in efforts to identify the necessary data to enable the estimation of effects and exposure levels needed to assess potential risks from these scenarios.

Detailed descriptions of each step in the process, *i.e.,* screening-level assessment to more refined evaluation of effects and exposure based on laboratory data, to higher tiered assessments involving semi-field and field studies can be found in Sections 1.2 to 1.4. Efforts to refine risk estimates are typically predicated on refining estimates of potential exposure and effects. For detailed descriptions of the studies to be undertaken to generate these data, refer to Chapter 7 (laboratory-based effect studies) and Chapter 8 (field-based effect studies). As with the risk assessment process itself, studies to determine potential exposure (see Chapter 6) and those examining effects in the laboratory (see Chapter 7) and under semi-field and full field conditions (see Chapter 8).

The flowcharts are used to depict a generic risk assessment process that was developed during the workshop. Two proposed processes distinguish between compounds applied as spray for which the worst case exposure may be expected through a direct contact of pollinators with spray droplets around the blooming period (**Figures 2** and **3**); and, products used as soil or seed treatments for which an exposure may occur as a result of the systemic properties of the compound or its degradates (**Figures 4** and **5**). (It is important to note that contact exposure to a systemic compound if it is applied via spray application, may also occur, *e.g.*, in the case of pre-bloom application. In this case, the reader may also find useful recommendations in the flowchart for soil/seed treatments.) Each box of these flowcharts is numbered and the nature of the data and reasoning behind each step of the process is provided below. As noted earlier, suitable trigger values for transitioning to higher levels of refinement are linked to risk management decisions and protection goals of individual regulatory authorities. The trigger values depicted in Figures 2-5 are generic. However, the more detailed but related risk assessment scheme in **Appendix 1,** which modifies the EPPO guidance (EPPO, 2010), contains trigger values currently used in the European regulatory process (EC, 2010). As stated in other parts of this document, it is not the intent of this document, or SETAC, to recommend and/or support any particular trigger criteria but rather to emphasize the role that these values play in an efficient risk assessment process.

Spray Applications

Figures 2 and **3** depict the risk assessment process for insect pollinators following the use of spray products. Each step (box) depicted in the flow chart is numbered and arrows depict the direction that should be followed in response to a "yes" or "no" answer. More detail regarding each of the steps is provided below.

The risk assessment process begins by asking whether exposure is possible (**Box 2a**); if exposure is not possible, then there is a presumption of minimal risk (**Box 6**). For sprayed applications, the screening level considers the worse case exposure assumption of a direct overspray to plants where bees are actively foraging. Potential effects of the chemical thus result from the overall effects of the direct spray on foraging bees.

As depicted in the left-hand side of **Figure 2**, at the screening level, potential risk to adult honey bees from spray applications, is assessed through calculation of an HQ (**Box 3a**). The assessor calculates an HQ by dividing the theoretical exposure, that is the application rate expressed in terms of weight per unit area (*e.g.*, grams active ingredient/hectare) by the most sensitive acute median lethal dose to 50% of the organisms tested, *i.e.*, the [dermal] LD_{50} value, derived from laboratory studies. If the HQ value passes a regulatory trigger value, then there may be a presumption of minimal risk to adult honey bees and the reviewer proceeds to assess possible impacts to non-Apis adults (**Box 4a**). To evaluate potential risk to *larval* honey bees, the assessor calculates a TER by dividing the most sensitive No Observed Effect level (NOEL) from the honey bee larval toxicity test by the theoretical maximum concentration in pollen and nectar (**Box 3b**). While several test designs currently exist to assess effects to larval, adoption of this step in a formal, regulatory process would require standardization of a particular test design. Possible test designs for lower-tier laboratory-based studies with larval are discussed in Chapter 7. If the TER value passes the trigger value, then a presumption of minimal risk to larval honey bees can be made and the reviewer proceeds to evaluate possible impacts on non-*Apis* larvae (**Box 4b**).

> *Default Exposure Estimates for Screening Level Analysis for Apis Larvae*: Although a theoretical maximum concentration has been established by some regulatory authorities for systemic products (*e.g.*, 1 mg/kg or ppm, EPPO 2010) no such exposure model or theoretical maximum concentration level has been formally set for sprayed products. Pesticide residues resulting from direct overspray on food items for birds and mammals can be estimated using a residue per unit dose (RUD) approach favored by Hoerger and Kenaga, 1972. In the most recent guidance produced by European Food Safety Authority (EFSA) (EFSA 2009[78]) a range of RUD values have been developed for different crops and food sources. Further research is necessary to both validate current screening exposure values used by regulatory authorities, as well as to develop RUD values, or other [screening] exposure models specific to pollinators.

The proposed risk assessment scheme also considers potential risks to non-*Apis* bees. At the screening level, risk to non-*Apis* bees is evaluated by employing effects data from honey bee acute oral/contact (LD_{50}) (**Box 4a** depicting the calculation of an HQ for non-*Apis* adults), and chronic larval honey bee toxicity (NOEL) test data (**Box 4b** depicting the calculation of a TER for non-*Apis* larvae). In cases where Tier 1 (screening-level) data on *Apis* bees are not sufficient to conclude low risks to non-*Apis* bees (*i.e.*, a trigger value for Apis species modified with an appropriate safety factor to account for inter-species variation), then it may be concluded that the substance does not pass the

[78] European Food Safety Authority; Guidance Document on Risk Assessment for Birds & Mammals on request from EFSA. EFSA Journal 2009; 7(12):1438. doi:10.2903/j.efsa.2009.1438. Available online: www.efsa.europa.eu

screening step. In this case, data from non-target arthropods (NTA) could be considered (**Box 4a and 4b)** as they may provide useful information on the choice of non-*Apis* species to be tested further if potential risk cannot be excluded on examination of the available NTA data. Participants in the Pellston agreed that NTA data, required by the EU, could be utilized as it typically includes toxicity estimates for the predatory mite (*Typhlodromus pyri*) and the parasitic wasp (*Aphidius rhopalosiphi*). Refined risk estimates for non-Apis bees would then require development of adult oral and/or contact LD_{50} values for the relevant non-Apis species and an HQ (*i.e*, application rate/LD_{50}) developed for adult bees (**Box 5a**). Similarly, where risk estimates do not meet trigger criteria for non-*Apis* bee larvae, then a NOEL for relevant non-*Apis* bees is necessary (**Box 5 b**) to calculate a TER. As with toxicity estimates for adult non-*Apis* bees, toxicity test methods would have to be developed for larvae of relevant non-*Apis* bees. If risk estimates for either adult and/or larval non-Apis bees are within regulatory criteria, then minimal risk is presumed (**Box 6**); however, if not, then the reviewer should proceed to higher-tier (refined) assessment methods depicted in **Figure 3** or consider risk management measures intended to reduce exposure (**Box 7**). As depicted in **Figure 2**, where risk management measures are imposed, the reviewer should then re-evaluate whether exposure to adults (**Box 2a**) and/or larvae (**Box 2b**) has been sufficiently reduced to presume minimal risk. Again, if minimal risk cannot be presumed, the reviewer should proceed through the screen using the revised exposure numbers based on the proposed mitigation.

The proposed refined risk assessment for sprayed products depicted in **Figure 3** begins by asking whether higher tier risk assessment is needed for honey bees (**Box 8a**) or for non-*Apis* bees (**Box 8b**). The screening level risk assessment is typically based on effects data on individual bees collected through laboratory studies. However, in refined risk assessments, the reviewer considers the results of semi-field and full field tests, which are typically conducted at the colony level rather than level of the individual bee. The refined risk assessment process therefore attempts to capture more realistic effects data as well as more refined estimates of exposure. For honey bees, effect estimates from semi-field studies (**Box 9**) or full field studies (**Box 10**) are used to determine whether maximum application rates result in effects. If minimal risk cannot be presumed from the results of semi-field studies, then the reviewer should consider full field studies where such studies can determine effects under more realistic test conditions (**Box 10**). In cases where full field studies do not result in risk estimates that are consistent with regulatory criteria, then the reviewer should conduct an analysis of uncertainties associated with the review process and whether possible mitigation specific to honey bees has been adequately considered (**Box 11**). As in the screening-level assessment, the impact of mitigation measures should be considered through the refined risk assessment process to address potential risk that is inconsistent with protection goals. After such an analysis, if risk estimates still do not meet regulatory criteria, then there is a presumption of significant risks (**Box 17**) to honey bees.

In the case of non-*Apis* bees, the reviewer assesses potential risks via data on non-target arthropods (**Box 12**) and determines whether there are actual significant routes of exposure which are not accounted for by the higher tier tests conducted using honey bees (**Box 13**) such as from contaminated nest material. If risk concerns to non-*Apis* bees cannot be minimized, higher tier effects testing discussed in Chapter 8 using non-*Apis* bees relevant to the specific potential route of exposure are then considered possibly first through a semi-field test (**Box 14**) with the option to extend the investigation to the full field level (**Box 15**). As with honey bees, the process and underlying assumptions/uncertainties associated with risk estimates should be carefully analyzed (**Box 16**) and the reviewer should consider possible mitigation measures specific to non-*Apis* bees. The potential effects of mitigation options must be considered at each of the steps within the refined process whether it is an Apis, or non-Apis analysis. If after this analysis, estimates are considered reasonable and potential mitigation measures cannot reduce potential exposure and potential risks, then the reviewer must presume significant risk to the non-*Apis* species considered.

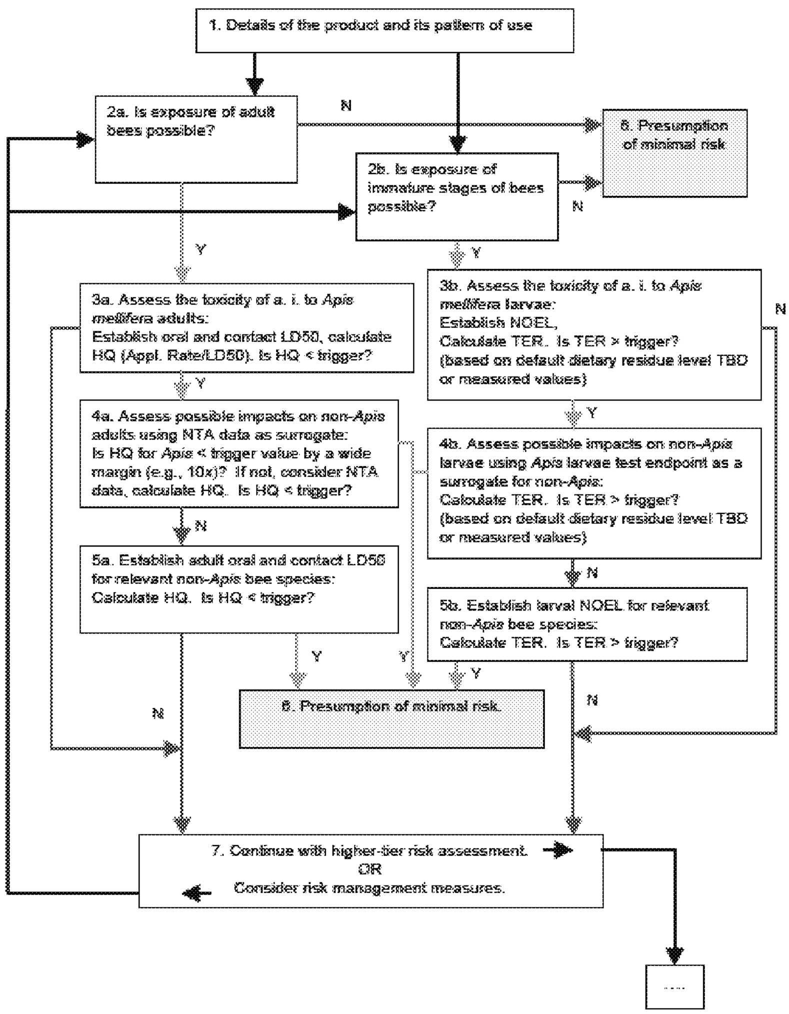

Figure [SEQ Figure * ARABIC]. Insect pollinator screening-level risk assessment process for foliarly applied pesticides.

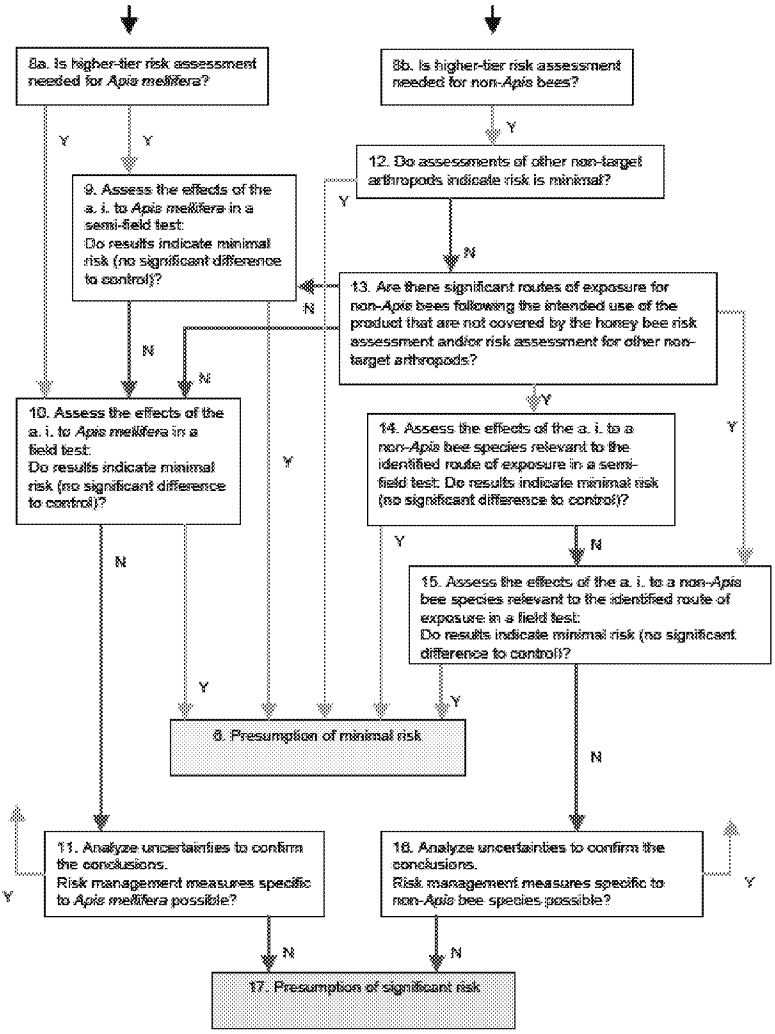

Figure [SEQ Figure * ARABIC]. Higher-tier (refined) risk assessment process for foliarly applied pesticides.

Soil and Seed Treatment Applications for Systemic Substances

Figures 4 and **5** depict the screening-level and refined risk assessment processes, respectively, for soil and seed treatment applied pesticides that are systemic in nature. Each step (box) depicted in the flow chart is numbered and arrows depict the direction that should be followed in response to a yes or no answer. More detail regarding each of the steps is provided below.

When evaluating potential acute risk to adult honey bees from soil or seed treatments[79] with systemic compounds, the assessor first asks whether exposure is possible to the adult (**Box 2a**) or immature stages (**Box 2b**) via systemic translocation of residues in plant material. If exposure to honey bee adults is considered likely, the review calculates a TER (**Box 3a**) using either an acute oral or contact LD_{50} value for honey bee adults. In Europe, a tier 1 TER is estimated by dividing a screening exposure estimate by the screening level hazard value. (Currently, EPPO has a proposed conservative default exposure value of 1 mg a.i./kg, relies on the default maximum concentration estimated in pollen and/or nectar from residues in whole plants, which for use with soil and seed treatments, see Chapter X for more discussion). If the risk estimate for the adult honey bees does not meet the regulatory criterion for low risk, then the reviewer should proceed to higher tier risk assessment (options to proceed with a 10-day adult test (**Box 4a**), or more refined studies) or consider risk management measures and reassess (**Box 8**). If the TER value for the adult honey bee meets the regulatory criterion for low risk, then the reviewer proceeds to evaluate potential impacts on non-*Apis* adults (**Box 5a**). Here the assessor may consider data on non-target arthropods. Where risk assessments for non-Apis bees do not meet the regulatory criterion for low risk (i.e., meets the regulatory criterion for low risk to *Apis* by a wide margin), then acute oral/contact LD_{50} values should be developed for non-Apis bees and a TER calculated (**Box 6a ~~5a~~**). As with honey bees, if the risk estimate does meet the regulatory criterion for low risk, then the reviewer should proceed to higher tier (refined) risk assessment (semi-field or field study) or consider risk management measures and reassess (**Box 8**).

[79] Although not specifically discussed at the workshop, treatments with systemic compounds can include tree trunk injections as well.

For larval assessments, the same process as that discussed for spray applications is followed (Boxes **3b**, **4b**, and **5b** of **Figure 4**). Additionally, the same process for higher tier (refined) risk assessment is used as discussed for spray applications. Participants of the Workshop noted the lack of information on potential exposure (nectar and pollen) related to trunk injection; and that further data are needed in this area (see Chapter 13). In the meantime, participants of the Workshop recommended that potential [screening] risks from trunk injection be estimated in the same manner as soil and seed scenarios. As discussed previously, risk assessment is intended to be an iterative process. At a screening level, when risk estimates do not meet decision criteria, (*i.e.*, where a presumption of minimal risk cannot be made), the conditions under which the estimated risks occur should be more closely examined. More detailed fate considerations (such as degradation), or use considerations (such as timing of application, or application intervals) should be considered before additional testing is required.

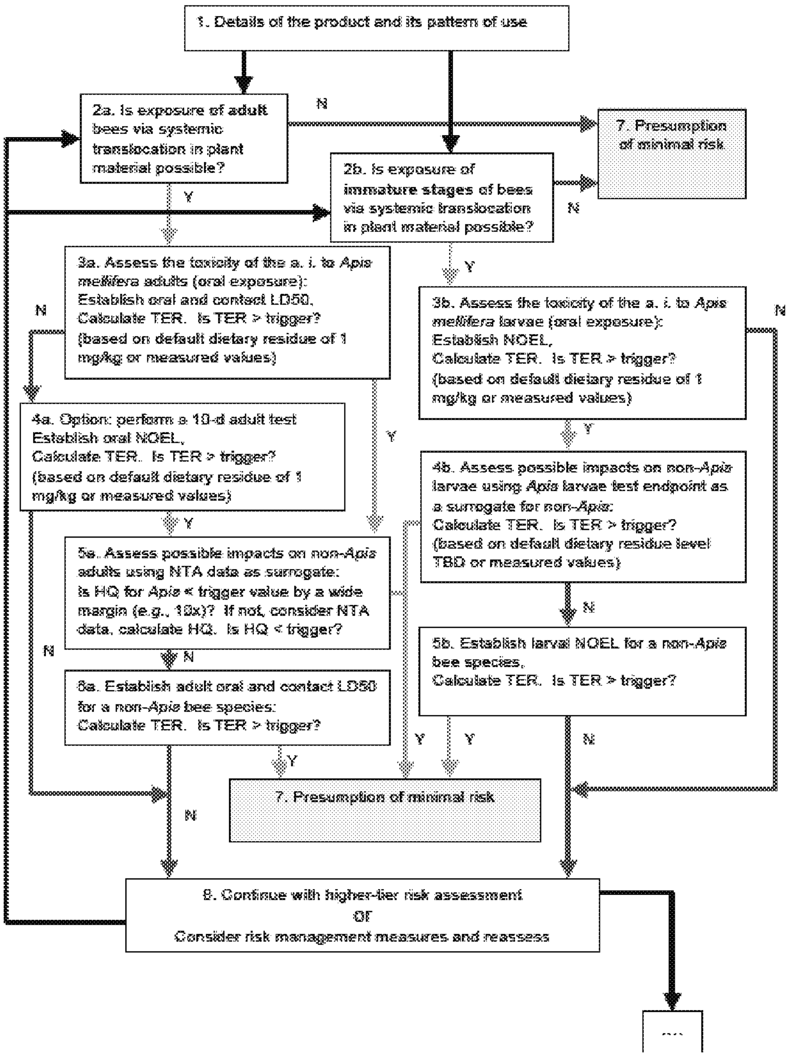

Figure [SEQ Figure * ARABIC]. Insect pollinator screening-level risk assessment process for soil and seed treatment of systemic pesticides. Note that this flow chart may apply for trunk injection as well, as modalities of exposure of pollinators are similar as for soil/seed treatments. For trunk injection however, further data are needed to appropriately describe the range of expected residue concentrations in nectar and pollen. As a consequence no default value is currently available for a quantification of the risk (Boxes 3a and 3b). A compilation of available data could be made, with a particular attention to the corresponding injection protocols as it varies with the active substance involved and the tree.

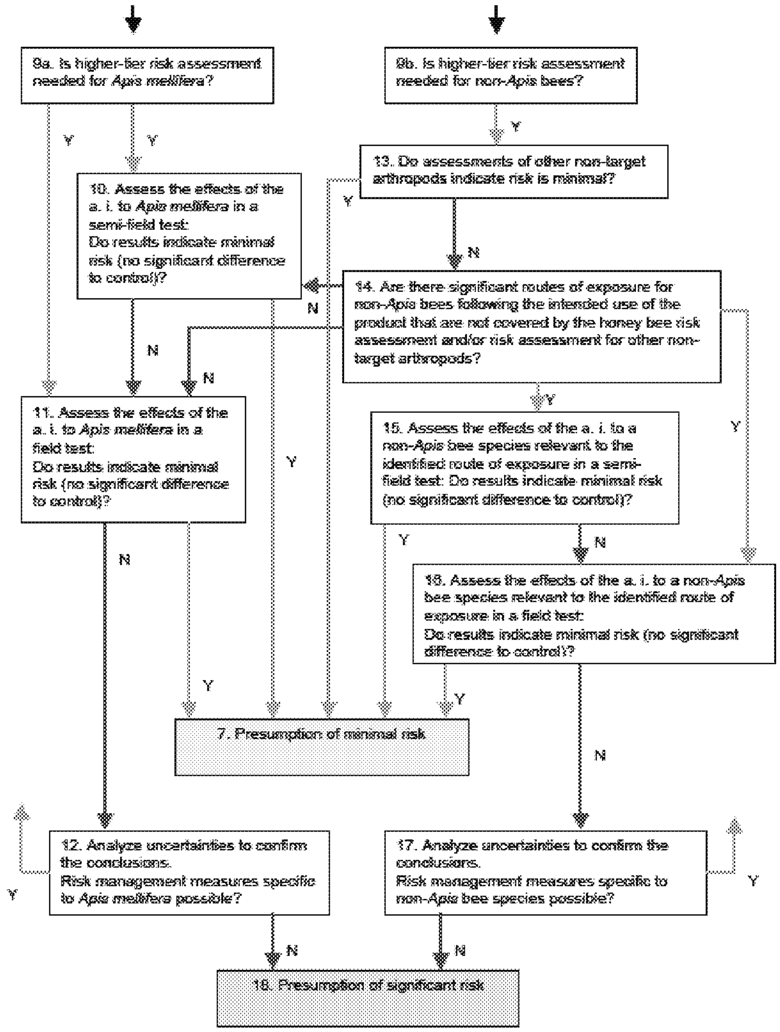

Figure [SEQ Figure * ARABIC]. Higher-tier (refined) risk assessment process for soil and seed treatment applied systemic pesticides.

Factors limiting uncertainty in the screening step

Screening-level assessments are typically based on conservative assumptions regarding both exposure and effects. In the case of honey bees, for example, at a screening level the EPPO system assesses risk based on the direct application (*e.g.*, spray) on foraging bees, which does not correspond to a good practice where sprayed treatments during bloom are applied when honey bees are not foraging. Further, other routes of exposure potentially exist (such as dermal contact with contaminated wax, or dietary exposure via contaminated guttation). Therefore, while the Participants of the Workshop acknowledge that not all routes of exposure are accounted for by the proposed risk assessment process, it is believed that the conservative assumptions used in the screen are protective for other potential routes of exposure.

Similarly, although mortality is the primary effect reported and used to generate LD_{50} values in acute toxicity tests, adverse effects on behaviour or growth are also reported. As discussed in earlier chapters, the extent to which sublethal effects occur and whether they ultimately affect assessment endpoints such as impaired survival, growth and reproduction remains an uncertainty for many compounds. However, since effects on growth or behaviour are most often associated with insecticides or acaricides which will also potentially affect acute survival, the majority of these compounds will be subject to higher tier risk assessment where the sublethal effects will be more thoroughly evaluated. In addition, other information presented in the data profile of a compound (such as mode of action, route of uptake, toxicity and effects on other types of terrestrial arthropods) should always be examined (EPPO, 2010), and integrated with the findings of the screening step as part of the overall risk assessment for honey and non-*Apis* bees.

The capacity of the screening-level assessment to properly screen substances of low risks from substances for which further assessment is necessary has been evaluated through a review of the honey bee kill incidents recorded in the United Kingdom survey network WIIS (Mineau *et al*., 2008). The Mineau *et al*. 2008 analysis supports the utility and efficacy of the tier 1 screening methodology, provided that considerations on the mode of action and use patterns are also kept in mind, as for any risk assessment process.

Refinement Options for the Risk Assessment

If a substance fails the screening-level assessment, it moves to a series of refinements in both exposure and/or effects data (see **Figures 2-5**). There are a number of options to further refine a risk assessment through a more in-depth description/characterisation of exposure and/or of effects. These options are described, regarding their possible methodologies, in previous chapters. As refinements progress, different TERs and RQs are developed.

In the deterministic risk assessment approach, the primary outcome of the [Tier 1] risk characterisation is the calculation of the risk quotient (RQ), or the Toxicity Exposure Ratio (TER) depending on the country/region where the assessment is being performed. Both the RQ and the TER are single number (point) risk estimates. In reality, risk is more complex and therefore, a single point estimate can be misleading. As a consequence, the assessor should characterize the RQ or TER with a description of the uncertainties, assumptions, strengths and limitations associated with the risk estimate. These sources of variability and uncertainty will largely be discussed during characterization of the exposure and effects and will include refinement options used in ultimately determining the RQ or TER. At the higher levels of refinement (*e.g.*, semi-field and field tests), the level of impact is directly measured in experiments that are intended to reproduce the operational conditions of subject pesticide product. In this case, TER and RQ values are no longer calculated.

Exposure is the first component of the risk to be examined to determine whether a risk assessment is needed, and the first to be explored to refine a potential risk. As a guide for proceeding through the levels of refinement, **Table 3** provides a summary of the relative importance of different exposure routes of *Apis* and non-*Apis* bees. The main exposure routes identified for evaluation in the screening-level assessment are oral intake of nectar and pollen, and contact exposure. While not all exposure routes are included in the screening-level (Tier 1) risk assessment (*e.g.*, wax, and drinking water are not evaluated at Tier 1); and, direct overspray is considered as the worst case [high-end] exposure, it is important for the assessor to consider additional exposure routes for higher tier risk assessment purposes (see **Table X** for potential exposure routes for different bees).

Table X. Likelihood of exposure to *Apis* and non-*Apis* bees from various routes.

Exposure	*Apis*		Non-*Apis*	
	Adult	Larvae	Adults	Larvae
Nectar	+++[2]	+	+ to +++[1]	+
Pollen	+ to +++	**[3]	+ to +++[4]	++ to +++
Water[a]	+ to ++	+[5]	+	+
Nesting Material[b]	+[6]	+[6]	+ to +++[6, 7]	+ to +++[8, 9, 11]
Exposure to Soil	-/+	-	- to +++	- to +++
Foliar Residues (contact and direct spray)	+++	_	+++	- to +++
Direct spray	+++[10]	-	+++[10]	-

[a] Collect water for cooling (evaporative cooling; take up into crop, regurgitate it and flap winds to distribute) and honey production; [b] [1]expected for parasitoid; [2]particularly for nurse bees; [3] bee bread; [4] (typhlodromus); [5] provided by nurse bees;[6] wax; [7]leaves and soil for cement; [8] leafcutting bees. [9] soil used to cap cells; [10] at flowering; [11] exposure to soil

Refinement options for spray applications

Refinement Options – *Apis* adults

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If the HQ for adult *Apis* exceeds the level of concern in the screening-level (Tier 1) assessment, then further information is required. Refinements can be made for exposure and/or effects, pending on the profile of the active substance and its residues.

For spray application, an option for refining exposure estimates is to move from the screening-level default values to product-specific field modelling or measurement data to better quantify exposure. If an application during flowering cannot be excluded, this option may have several levels of refinement such as consideration of the interval between application and blooming and the expected level of residues to which bees could be exposed, for either modelled or measured estimates of refined exposure. Measurements of actual exposure may be achieved by use of the existing residue data set, *e.g.*, magnitude of residue studies on what may be considered vulnerable crops, or by implementing tunnel and/or field residue studies to appreciate the level expose in treated crops and considering different modalities for the period of treatment.

While most field testing (semi-, or full-field) generates data on both exposure and effects, they may also be pursued with an exclusive aim of providing realistic exposure estimates which, in turn, can be compared to effect measurements (*i.e.*, toxicity test endpoints). In this case, it is important that data generated from the field test is recorded so that it may be directly compared to the ecotoxicity data (*i.e.*, the results and endpoints are expressed in the same units and represent comparable measures of exposure).

With respect to residue concentrations in nectar, pollen (or foliage where appropriate) the reviewer should consider the 90^{th,} percentile of measured concentrations as a conservative measure of exposure. However the decision to use a 90th percentile or other value ultimately depends on the data set. If data are derived from only a single test on one crop, then a specified percentile, *e.g.*, 90th percentile, should be sufficiently vetted to reflect the uncertainty and variability as is frequently done in support of probabilistic approaches. If several trials have been undertaken, or data are derived for several crops, then a mean or a lower percentile may be more appropriate and would achieve the same level of protection.

The initial test(s) to measure the effect of a compound is a lethality test consistent with relevant life stage and exposure route (*e.g.*, oral LD_{50}, or larval toxicity test). As effects tests become more refined, they incorporate more environmentally realistic conditions and begin to reflect both intrinsic toxicity and potential enhancing/compensatory effects, related to environmental conditions~~.~~

To further refine the toxicity end-point, additional *Apis* studies that could be relevant for the adult life stage include:

- 10-day feeding study (adult survival);
- toxicity of residues on foliage study;
- Semi-field data ;
- Field data.

A description of the studies that may be appropriate is found in Chapter 8; these studies are discussed briefly below.

The 10-day adult study is an extension of the standard laboratory oral exposure method (OECD 215). The test exposes adult bees for a period of 10 days and measures lethal effects after ingestion of product over the entire test duration. A NOEL is derived, that may be used similarly as a LD_{50} in RQ calculations. Because this test only addresses oral exposure, it is not sufficient to address the uncertainties associated with sprayed compounds and is actually considered to be useful when refining estimates of effects for systemic soil/seed treatments. Currently there is no internationally recognised guideline for the 10-day feeding study nor for the larval toxicity testing in the laboratory; these tests would need to be developed and validated before [formal] inclusion in to a regulatory risk assessment scheme. The endpoint from a 10-day feeding study could be compared to either the default (screening-level) exposure concentration, or to refined exposure concentrations based on field measurements, both expressed in mg a.i./kg.

The EPA foliar residue toxicity study is more representative of the conditions of exposure for bees after a spray event. This study is designed to evaluate the effects from exposure to dry and aged residues (3, 6 and 24 hours) and thus provide information on the level of bioavailability and length of residual hazard of the substance.

As discussed in Chapter 8, semi-field studies reproduce even more closely the conditions of exposure of bees in a treated crop. The test provids information on colony health based on bee survival and development related to actual field application parameters. (Semi-field tests can be pursued with pollinator attractive crops treated at flowering (e.g., Phacelia), and/or pursued with the actual target crop when a treatment at flowering cannot be excluded. Semi-field and field tests can also provide additional information the can refine an assessment such as information on potential exposure outside the flowering period of the crop, or through spray drift onto flowers in vegetated areas, or onto flowering weeds within the crop (e.g., in orchards). Finally field tests may allow the evaluation of the efficacy of certain risk mitigation measures to limit exposure such as reduced application rates, modifying application intervals.

Refinement Options – *Apis* Larvae

As for the adults, an option for refinement of exposure is to move from the screening-level default values (*e.g.*, application rate or default consumption rate), to product-specific field modelling or actual measured residues (*e.g.*, in pollen and nectar) to better quantify exposure of larvae. The same considerations with regard to the generation and use of these data apply (see 2.1.1.1).

Additional *Apis* studies that could be relevant for the larval or immature life stages include:

- Brood feeding study (brood development[80]);
- Semi-field data;
- Field data.

[80] For example the method of Oomen PA, de Ruijter, A, and Van der Steen J (1992) EPPO Bulletin, 22, 613 - 616.

The brood feeding study aims at evaluating the effects on the development of the honey bee to derive a NOEC. This NOEC can then be compared to either default (screening-level) concentration estimates or to refined concentrations based on field measurements.

The semi-field and field tests are similar with respect to measurement of effects on adults (see Section 2.1.1.2) and both can provide information on colony health and brood development. As discussed elsewhere, field studies typically do not lend themselves to producing a dose/response relationship (*i.e.*, a NOEC or LOEC) due to scale and logistical reasons. Consequently, the assessor must evaluate whether the study results indicate a minimal level of risk exists (for example, no significant difference between test and control plots) Levels of refinement of effects beyond the laboratory and semi-field may involve assessing impacts of the formulated product in full field tests. Further discussion and guidance on semi-field, and field tests can be found in Chapter X, and discussion and guidance on brood tests can be found in Chapter X these tests may be found in Chapter 8 (Effects).

Refinement Options – Non-*Apis* adults

Non-*Apis* bees may differ from honey bees in their exposure and sensitivity to plant protection products (Devillers *et al*. 2003).,. Most non-*Apis* bees are solitary, with single females that forage for pollen and nectar to feed their offspring, construct their nests, and lay eggs (see introduction to non-*Apis* biology). The death of a foraging female implies the cessation of her reproduction (Tasei 2002). In comparison, when a [honey bee] colony looses female workers, the loss may be compensated by the colony, *e.g.*, by engaging inactive workers (Robinson 1992) or through reduced foraging age (Winston & Fergusson 1985), so the colony may continue to develop as a viable unit. For bumble bees some colony recovery is also possible (Schmid-Hempel & Heeb 1991). However, the death of the bumble bee queen, in the spring signifies the death of the potential colony that would be formed (Thompson & Hunt 1999).

In comparison to honey bees, the life-history traits of non-*Apis* bees such as sociality and nesting behavior result in a greater importance of certain exposure routes. . For example alfalfa leafcutting bees (*Megachlie rotundata*) may be more exposed to foliar residues (George & Rinker 1982), ground nesting bees to soil residues and larvae to pollen residues. These differences mean that representatives of the main non-*Apis* groups for which we have sufficient knowledge should be considered for higher tier testing of a plant protection product for bees when a risk cannot be excluded. Where non-*Apis* species are chosen for higher tier evaluation they should be amenable to experimentation, provide reliable and reproducible results and the methods should comply with internationally recognised and validated guidelines (e.g. OECD test guidelines). The exact choice of species may be selected based on the proposed use of the product and on regional [species] considerationss; however, it should be possible to extrapolate from "standard" species (*e.g.*, *Bombus* sp.) to reduce the need for unnecessary testing.

Participants of the Workshop proposed that higher tier testing could be conducted with social non-*Apis* bees from the tribes *Bombini* and *Meliponini* and solitary bees that are ground nesting and cavity nesting (**Table 4**). While techniques exist for both laboratory and field/semi-field tests for *Bombini* spp. (*B. terrestris* and *B. impatiens*)s (for review on *Bombus* spp. see van der Steen 2001) standardization is needed. Similar tests are in development for *Meliponini* spp. Sufficient knowledge exists of the ecology of the *Bombini* and *Meliponini* tribes to be able to predict the main exposure routes (see Chapter 6, Exposure). For cavity nesting solitary bees (*Osmia lignaria* and *Megachile rotundata*), laboratory and field/semi-field tests have already been successfully implemented (Abbott *et al*. 1998; Alston *et al*. 2007; Ladurner *et al*. 2008). For ground nesting bees, while primary exposure routes can be predicted, there are not yet the techniques to perform standardized tests on them in the laboratory or the field. Until such techniques are available, the solitary cavity nesting bees may sufficiently represent "solitary non-*Apis*" as a group, taking into account that for ground nesting species, soil residues may play a more important route of exposure. Note however that even for Bombinae and Meliponinae no validated or internationally recognised test protocols exist which currently limits their inclusion into a risk assessment scheme at this point in time andfurther research is needed.

Exposure

Similar to the refinement process for adult honey bees, the option for refinement of exposure to adult non-*Apis* bees is to move from the screening-level default values to product-specific field modelling or measurement data to better quantify exposure of non-*Apis* larvae. Table 3 provides further guidance on the specific conditions of exposure for non-*Apis* species. The same considerations with regard to the generation and use of these data apply (see Section 2.1.1.1).

Effects

As discussed previously, at a screening level, the adult *A. mellifera* is used as a surrogate for non-*Apis* species. To take into account interspecies variation and the different life-history characteristics between that of the honey bee and non-*Apis* bees, a safety factor may be built into the level of concern (LOC) for *Apis* (participants of the Workshop considered a 10x factor). Then as illustrated in the flow chart, if the HQ is less than the adjusted non-*Apis* LOC, then risk is presumed as low for non-*Apis* species; and, where it is not, further refinement of the ecotoxicity data may be undertaken.

When available, non-target arthropod data may be considered at this stage, as it may provide relevant information on effects (and route specific exposure) to non-Apis species see **Table 3**.

The nectar feeding parasitoid *Aphidius rhopalosiphi* and the soil-dwelling beetle *Aleochara bilineata* are among the most sensitive of the non-target arthropods tested under the European ESCORT scheme (Candolfi *et al*., 2001). Adult parasitoid such as *Aphidius* also feeds on nectar which makes of it a good representative for exposure conditions of pollinating species. Similarly, approximately 70% of non-*Apis* bees are ground nesting (Michener 2000) and the ground-dwelling beetle *Aleochara bilineata*,which is tested for sensitivity to plant protection products through sand/soil under the European ESCORT scheme, such that data from its contact toxicity tests may be considered informative for ground nesting bees. In the cases where a refined risk assessment has been triggered for non-Apis adults, the data set developed in the European process may contain information on up to 8-10 species in the laboratory and more when semi-field/field testing have to be undertaken for refined risk assessment purposes (Candolfi *et al.*, 2001) (**Table X**). In these cases, inventories of the species identified in the crops tested may also be useful information in evaluating whether a particular concern is raised for non-*Apis* species which would need to be investigated further.

Table X: testing methodologies developed for the risk assessment to Non-Target Arthropods developed in European process of evaluation of pesticides (Candolfi et al., 2001)

Testing scale	**Species (and stages tested)**
Tier I Laboratory: artificial substrate	*Aphidius rhopalosiphi (adults + life cycle)* *Typhlodromus pyri (protonymphs + life cycle)*
Tier II (extended) Laboratory : natural substrate	*Aleochara bilineata (adults + life cycle)* *Aphidius rhopalosiphi (adults + life cycle)* *Chrysoperla carnea (larvae + life cycle)* *Coccinella septempunctata (larvae + life cycle)* *Orius laevigatus (nymphs + life cycle)* *Pardosa sp. (adults)* *Poecilus cupreus (adults)* *Trichogramma cacoeciae (adults + life cycle)*
Semi-field	e.g. *Poecilus cupreus (adults)*

	Methods can be adapted for many species
Field	Arthropods (populations and communities)

If relevant NTA data cannot be found then the assessor may consider selection of an appropriate non-*Apis* species for employment in acute laboratory testing (**Table 4**, see Chapter 7, Hazard, Laboratory). The choice of species will be guided by which tests have been developed (**Table 4**). Data from residue studies and field measurements (i.e., pollen, nectar, foliage and soil) **Table 3** will inform study design with respect to exposure routes and therefore which non-*Apis* group is most appropriate to test (see also Chapter 6, Exposure). For example a plant protection product with high foliar residues would suggest that higher tier testing should be performed on alfalfa leafcutting bees (*Megachile rotundata*) if such bees will visit the crop to harvest nesting material and exposure may occur.

Based on the work underpinning the ESCORT approach, the dose/response data and decision making process are considered to be protective also of the pollination function of non-*Apis* bees in as far as they consider adult mortality and fecundity of non-target insect species (Alix *et al*., 2011). Following this approach, if the RQ derived from non-target arthropod (NTA) data (including extended laboratory data if available) does not exceed 2, then assessment criteria are considered to be met and the assessment does not need to proceed further (see also Section 1.3.1 for a comparison of the outcome of the screening steps for the honey bee, non-*Apis* as estimated from the honey bee, and NTA). Other available NTA data can be used provided test data meet tests for relevance and reliability.

Alternatively as shown in the flow chart (**Figures 2-5**), non-*Apis* specific test data for adult contact or oral toxicity can be generated. These data are likely to be in the form of an LD_{50} (µg/bee) with derivation of an HQ as for adult *Apis*. Based on the European HQ approach, in this case for assessment criteria to be met, the HQ must not exceed the trigger value, in which case the assessment does not need to proceed further. The most appropriate trigger value to be used may be discussed further and additional safety factors may be considered to account for interspecies variability among non-Apis species intended to be protected by this HQ calculation (see also section 1.3.1 for a comparison of the outcome of the screening steps for the honey bee, non-*Apis* as estimated from the honey bee and NTA).

Levels of refinement of effects beyond the laboratory and semi-field may involve assessing impacts of the formulated product in field tests. Guidance on the type(s) of test(s) may be found in Chapter 8 (Effects). The field or semi-field tests will monitor behaviour and quantify bee mortality and fecundity of one or several selected non-*Apis* species (see Chapter 8 Hazard, Field) likely to be encountered in the crops to be treated with the product. Additionally, non-*Apis* solitary bees allow the impacts of a plant protection product to be assessed at the population level. Also cavity nesting bees (*e.g.*, *Megachile rotundata* and *Osmia lignaria*) and *Bombus spp.* have shorter forage distances compared to the honey bee and are therefore easier to control foraging on the crop to which the plant protection product is applied in experiments (see Chapter 8 Hazard, Field for methods and advantages of field tests on non-*Apis* bees). **Table 5** at the end of this section highlights the availability of laboratory and field tests for representative groups of social and solitary non-*Apis* bees.

Risk Characterization (Estimation)

As for the honey bee, different outcomes for the risk characterization may be expected based on the refinements undertaken.

For both Apis and non-Apis assessments, when higher level field data are developed, the results are not expected to be applied in a TER and/or quotient context, but may be used directly in the risk assessment. Again, mitigation of potential risk remains as an important pathway to meeting protection goals whether at the screening or higher tier steps of the analysis.

Refinement Options – Non-*Apis* Larvae

Exposure

A general description of exposure sources for non-*Apis* species (immature stages) is provided in **Table 3**. Where honey bee larvae are exposed primarily in larval food which is processed pollen (see Sec XXXX), non-Apis larvae are typically fed unprocessed pollen which could potentially carry a higher residue load. This should be considered when generating a refined [exposure] analysis for non-Apis species since it may have implications on the origin of the pollen to be collected for analytical purposes.. For example, pollen sampled in the field or from loads taken at the hive entrance (pollen traps) or from forager bees directly may represent concentrations found in unprocessed food sources. Concentrations of residues from pollen sampled from within hive food stores or from larval cells could be more relevant to honey bee larvae.

Non-*Apis* larvae may also be exposed through contact with the pollen and nectar food provision in the nest. In addition the larvae of ground nesting bees and cavity nesting bees which separate their nest cells with soil (for example, *Osmia lignaria*) may come into contact with soil applied plant protection products. Similarly the larvae of leafcutting bees may come into contact with a plant protection product through residues on the foliage used to construct its nest (see Chapter 6, Exposure). Non-Apis species have various sources of exposure (e.g., treated soil, or nesting material). Refining potential exposure estimates to non-Apis bees to account for the different exposure sources would be difficult to achieve in a specific exposure test. In this case, it would be more appropriate to refine potential exposure and risk through a semi-field or field study (see Chapter 8).

Effects

As discussed earlier, honey bee larvae are proposed as a surrogate for non-*Apis* larvae as there is currently no formal guideline established for testing non-*Apis* larvae.

As the assessor moves through the proposed process, they may consider NTA data, if available, which may provide relevant information to refine potential risk to non-Apis species (Candolfi *et al*., 2001). These tests measure a wide range of endpoints including both juvenile and adult survival, fecundity or larval development and predation depending on the species being tested (see **Table 4**). All NTA tests (are designed to detect very small changes in sublethal endpoints, and therefore, an understanding of an application rate that may result in low impact on growth and/or fecundity or other sublethal parameter may be derived. Beyond laboratory tests, refining an understanding of potential effects to non-Apis larvae may involve field tests with formulated products see Chapter 8). While field and semi-field tests have not been specifically developed for ground nesting bees, monitoring, if possible, of cavity nesting bees (particularly *Osmia* spp. which can partition their nest cells with mud) through field or semi-field tests may provide information on some of the larval exposure routes that are unique to non-Apis species. .

Table X at the end of this section highlights the availability of laboratory and field tests for representative groups of social and solitary non-*Apis* bees.

Risk Characterization (Estimation)

If effects data on non-Apis larvae have been generated and provide a NOEC, then this value could be used as in the TER calculation. Both default and refined exposure estimates may also be used in the TER calculation. As noted in the flow diagrams, should this assessment indicated risks that are not consistent with protection goals, then, either mitigation measures may be considered or the assessment may proceed to further refinement.

Again, when data are generated from field tests, the results are not expected to be applied in a TER (quotient-based) context, but rather incorporated directly into a risk assessment.

Table X. The availability of laboratory and field tests for representative groups of solitary and social non-*Apis* bees (see laboratory and field chapters for detailed protocols).

Study Type		Solitary		Social	
		Cavity-nesting (tube, wood)	**Ground-nesting**	**Bombini (bumble bees)**	**Meliponini (stingless bees)**
Laboratory	**Adult**	Species available – tested *Megachile rotundata* (Huntzinger *et al.* 2008; Scott-Dupree *et al.* 2009), *Osmia lignaria* (Ladurner *et al.* 2005; Scott-Dupree *et al.* 2009), (temperate, north) – in development *Xylocopa spp.* (Brazil)	Limited availability of tested species – *Nomia melanderi* (Johansen *et al.* 1984; Mayer *et al.* 1998)	Species available – tested *B. terrestris* (for a review see Thompson 2001), *B. impatiens* (Scott-Dupree *et al.* 2009; Gradish *et al.* 2011b) (needs standardized guidelines of currently used lab bioassay and microcolony assays)	Species available – tests in development (Macieira & Hebling-Beraldo 1989; Valdovinos-Nunez *et al.* 2009) (tropics)
	Larva	Species available – tested *M. Rotundata* (Peach *et al.* 1995; Gradish *et al.* 2011a, Hodgson *et al.* 2011), *O. Lignaria* (Abbott *et al. 2008*) – in development *Xylocopa* spp. (Brazil)	Not yet investigated	Species available - tested *B. terrestris* (for a review see Thompson 2001), *B. impatiens* (Gradish *et al.* 2010; Gradish *et al.* 2011b) (needs standardized guidelines of currently used lab bioassay and microcolony assays)	Species available – tests in development (tropics)

Field	**Semi-field**	Species available – tested *M. rotundata* (Johansen *et al.* 1984, Tasei *et al.* 1988, Mayer & Lunden 1999), *O. Bicornis* (Konrad *et al.* 2008), *O. lignaria* (Ladurner *et al.* 2008), (temperate, north)	Can be developed	Species available – tested B. *terrestris* (Tasei *et al.* 2001), *B. impatiens* (Gels *et al.* 2002) (needs standardized guidelines)	Species available – tests in development (tropics)
	Field	Species available – tested *M. Rotundata* (Torchio 1983), *O. lignaria* (temperate, north)	Limited availability of tested species – *Nomia melanderi* (Mayer *et al.* 1998)	Species available – tested B.*terrestris* (Tasei *et al.* 2001), *B.impatiens* (needs standardized guidelines)	Species available – tests in development (tropics)
Exposure **Pollen, nectar, foliar, soil**		Can be developed (for pollen provisions in the field see Abbott *et al.* 2008; for foliar resides see George & Rincker 1982)	Not yet investigated	Can be developed (for pollen see Morandin *et al.* 2005)	Can be developed

Soil or Seed Treatment Application for Systemic Substances (also including trunk injection)

Exposure Characterization – Adult *Apis*

While there are differences in the screening-level assessment for calculation of HQs/TERs between sprayed pesticides and systemic substances, the general approach to refining the risk assessment for systemic applications is largely similar to that for spray applications. The primary difference is that for systemics exposure levels via contact are largely below that which may be encountered via oral. **Table 3** should be consulted for exposure routes specific to non-*Apis*. For example, for systemic compounds, leafcutting bees may be exposed orally through the foliage used to build its nest. The most appropriate way to explore this further is through simulating exposure conditions in a semi-field or a field test (see Chapter 8).

As stated earlier, for trunk injection, further data are needed to appropriately describe the range of expected residue concentrations in nectar and pollen that may be used in a risk estimate for this application method. In the future, a compilation of available data could be made, with a particular attention to the corresponding injection protocols as it varies with active ingredient and tree species.

Effect Characterization – Adult *Apis*

If risk cannot be excluded at the screening-level assessment, then a tier 2 assessment, based on the 10-d NOEL for young adult honey bees, can be conducted. The 10-day test is appropriate measure to refine the acute effects endpoint employed in the tier 1 assessment (i.e., oral LD50). The 10-day test may be run based on the default maximum concentration estimated in pollen and/or nectar, or on refined measured values, if these are available (see section 2. Refinement Options for the Risk Assessment for more detail on the options). In this case if the TER value exceeds triggers, then one may reach a presumption of low risk to adult honey bees from soil/seed applications. If viable exposure routes exists for the immature stages of either honey bees or non-*Apis* species, (e.g., through contaminated pollen or bee bread), then the approaches for refinement to soil/seed scenarios is similar as that for spray treatments (Sections 2.1.2.2 and 2.2.2.2). For higher tier testing (semi-field and field testing) protocols may be adapted to reflect crops grown from coated seeds or to products applied on/to soil, or for trunk injection. These tests may include monitoring of effects at sowing if measurements from potential exposure via seed dust (if it cannot be excluded or mitigated), or measurements of potential exposure to non-*Apis* species that might frequent the soil.

Risk Characterization (Estimation)

Similar principles as for spray application do apply for soil/seed treatments and trunk injection.

Conclusions on the Risks and Recommendations

Concluding a risk assessment is probably the step that best reflects how case-related the risk assessment process can be. Conclusions could be very brief and simply indicate that, under the assessment that was conducted (ie., whether it was screening level or a higher-tiered assessment) the use of the product meets the protection goals of the respective regulatory authority. However, where a refined risk assessment was triggered, there is a need to clearly express the following information in the conclusions:

- the concerns were identified at the screening step;
- whether/what concerns were identified in higher tier assessments(s)
- whether results of the higher tier assessment, addressed potential risk concerns;
- whether/which mitigation measure were considered at different levels of analysis, and whether the mitigation measure(s) reduced potential risks to an acceptable level;
- whether, despite higher tier analysis, all avalialble lines of evidence, and consideration of mitigation measures, potential risks remain; and
- remaining uncertainties [if any] in the risk assessment.

Risk assessment conclusions should give particular emphasis to the three following areas which are essential in providing appropriate information to risk managers for decision making. These are:

- the appropriatness of the available data to assess potential risks posed by the subject compound, or product.
- defining the use parameters required in order that the protection goals to be met;
- characterization of any potential risks, including remaing uncertainties from a lack of data or deficiencies in the existing data.
- where refined risk analysis indicates risk, characterization should be provided that regarding the growth, reproduction or survival of the organism (colony/population); possible interaction with plants and ultimately with stated protection goals.

Risk assessment conclusions should characterize the possibility of risk based upon the available lines of information (data, monitoring information, incidents, etc.). Characterization should include discussion of potential risk to of of the any specific life stages or casts. In certain cases, exposure considerations should focus on gathering more refined data such as:

- characterizing spray drift onto adjacent crops/vegetation that are attractive to bees;
- characterizing exposure to residues that could reach pollen/nectar of the crop for pre-flowering applications of systemic compounds, and of mobilization of soil residues in rotational crops (where relevant).

The risk assessment should be able to address the meaning of effects, *e.g.*, a temporary increase in the mortality of foragers, avoidance of a treated crop over the first days post treatment, *etc*. Field and semi-field studies allow for the monitoring of colonies/populations over long periods and assessment endpoints can be measured to address these concerns. Unresolved issues over time (temporal) or spatial scale could also be addressed through modelling tools when sufficiently developed[81]. Where uncertainties are related to "borderline" or "minor" effects and do not strictly compromise the protection goals, they may be appropriately addressed by implementing a monitoring study. The advantage of monitoring in this respect is to verify that protection goals will be met under conditions of agricultural practice in the real environment without any effort to control of other stress factors.

If a decision is made not to authorize a use, then it must be based on the evidence that protection goals for a particular product cannot be met. The inability to meet protection goals implies that based upon the available lines of evidence and higher tiered analysis, neither exposure (or hazard) can be reduced or avoided. and resulting risks will compromise protection goals. It is the responsibility of both the risk assessor and risk manager to discuss the conditions of the assessment and explore management options, if these are warranted. Both the assessor and manager should consider whether information exists that would determine whether all option to refine or mitigate potential risks have been explored before a final decision is reached.

Recommending risk mitigation measures

Please see Chapter XX for a discussion of risk mitigation with respect to pollinators.

Additional Tools in Support of Risk Assessment and to Inform Risk Management

Any tool that may help to better interpret data (*e.g.*, statistical and mathematical tools) should be used and in particular when higher tier data have been generated. In addition to these tools which now often enter in the usual package of risk assessment, modeling and landscape management approaches are possibly the most promising ones to further support both risk assessment and risk management provided these tools are sufficiently vetted and validated against measured data.

[81] Modeling tools have been successfully developed in other areas of ecotoxicology for that purpose.

Modeling Tools

Modelling tools may provide insight on uncertainties identified in a risk analyses that cannot be readily addressed by laboratory and/or field studies. Modelling population dynamics, may be used to simulate the fate of the population or colony over years of exposure to the product, and/or at a wider scale than the field, and may have the potential to address generic questions such as colony-level implications from individual-level effects. Development of models for honey bees and non-*Apis* bees could thus address general questions such as:

- What level of mortality or brood loss is of minimal consequence at the colony or population level?
- What magnitude and frequency of effects on adult survival and brood success is required to put the viability of a honey bee colony at risk?
- How do these thresholds vary according to season?

Answers to these generic issues are of great interest in conducting and interpreting risk assessments but also in support of decision making. The potential usefulness of modelling tools is discussed in more detail in Chapter XX.

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Chapter 11 Ecological Modeling for Pesticide Risk Assessment for Honey Bees and Other Pollinators

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Introduction

Current pesticide risk assessment for honeybees (*Apis* mellifera L.) is based on laboratory tests and on semi-field and field studies. Risk assessment schemes focus on quotients of the hazard imposed by a compound and the predicted exposure to this compound in the field. Depending on this quotient, in a tiered approach individual larvae and adults or entire experimental colonies are tested under confined or open field conditions. This scheme provides a wealth of important information for risk assessment. Test methods, experimental designs, standardization, and new and comprehensive endpoints are under continuous development and will help improve the efficiency and reliability of current risk assessment schemes.

There are, however, a number of questions relevant for ecological risk assessment that cannot be fully answered with laboratory and field studies. Ecological risk assessment tries to determine the risk of „unacceptable" adverse effects on populations but it remains unclear how to establish whether an effect is unacceptable or not (Hommen et al. 2010). Tests focusing on the individual organisms deliver information on mortality or sub-lethal effects under laboratory conditions, but leave uncertain what these effects mean at the population level, for example whether or not they impair the ability of the entire colony to persist, to cope with other stressors, and to reliably provide services such as honey production and pollination.

To assess effects on natural populations in general, ecological factors such as adaptive behavior, population structure, density dependence, exposure patterns, landscape structure, and species interactions need to be taken into account (Forbes et al. 2009). Additionally, for social insects like honeybees, it needs to be considered that the reproductive unit is not the individual worker bee, but the entire colony and its queen. The colony and its functioning can be considered as a complex net of buffer mechanisms that has evolved to increase the fitness of the queen. The loss of individual worker honeybees might thus be less significant than in solitary species. [Though beekeepers may see it differently – but only if honey harvest is impaired) On the other hand, buffer mechanisms have only certain capacities. We cannot easily know these capacities and how they are affected by other stressors such as varroa mites (*Varroa destructor*), viruses, changes in landscape structure, or beekeeping practices.

Semi-field and field studies allow inclusion and manipulation of some ecological factors, but certainly not all of them in all possible combinations within experimentally controlled conditions. They are expensive, time-consuming, require interpretation by experts, and may still be inconclusive as sufficiently controlled conditions are rarely achievable under field conditions. In addition, behavioral responses of colonies and foraging bees show large variations that can make it difficult to obtain any „clean results", i.e. clear effects of a certain factor on honey bee populations.

Ecological models provide a tool to overcome limitations of empirical studies. They are widely used in theoretical and applied ecology because ecological systems are usually too complex, develop too slowly, and cover areas that are too large to be studied solely via controlled laboratory or field experiments. In the context of regulatory risk assessment, ecological models are often grouped with individual-level models addressing toxicokinetics and toxicodynamics (TK-TD) or dynamic energy budgets (DEB) to „mechanistic effect models" (Grimm et al. 2009). This terminology was introduced to distinguish these models, which simulate processes related to effects of pesticides on organisms and populations, from fate models which focus on the fate of pesticides in water and soil, and from statistical or empirical models, which establish correlative, but not causal, relationships between factors. Ecological models can address all levels of organization beyond the individual, but in ecological risk assessment usually focuses on populations (Schmolke et al. 2010a, Galic et al. 2010). In this chapter we give a brief introduction into the rationale and approaches of ecological modeling of population dynamics. We present an example model to demonstrate the potential insights that can be gained from such ecological models, summarize current modeling practice and describe recent attempts to establish good modeling practice, which is needed to make mechanistic effect models applicable for regulatory risk assessment. We then provide an overview of existing models of honey bee colonies and give recommendations for the potential use of these models for pesticide risk assessment. Although this chapter focuses on honeybees, we will also briefly discuss how ecological modeling could support risk assessment of non-*Apis* pollinators. We will not discuss models addressing ecosystem services, which are important but belong to a different category of models and address different questions (Kevan et al. 1997, Williams et al. 2010).

Example model: common shrew

The following example model demonstrates how well-tested population models can be used to extrapolate the effects of toxicants observed at the individual level to the population level while considering different exposure patterns and landscape structures. Since such a demonstration does not yet exist for honeybees or other pollinators, we use a model of the common shrew (*Sorex araneus* L.). Wang and Grimm (2007) developed an individual-based population model of this species, which is a common insectivore. The purpose of the model was to explore the population-level consequences of acute mortality induced by pesticides.

The key behavior of the common shrew, which determines its response to heterogeneity in habitat quality and to the local density of conspecifics, is territoriality, i.e. the aggressive defense of a certain area to secure resources and habitat. Therefore, the model is spatially explicit and represents each individual of the population, its life cycle, and its territorial behavior. The habitat consists of hexagonal units of 5 m diameter which are characterized by habitat type (e.g., grassland or hedge) and level of food resources on a given calendar day. Individuals are characterized by the variables age, gender, developmental stage (lactating offspring, subadult, adult), fertility (fertile, infertile; applies to females only), pregnancy, and home range. Home ranges are a set of habitat units occupied by a certain individual.

The processes of the model comprise development, mortality, reproduction, home range dynamics, dispersal, and mating. The model proceeds in daily time steps and covers an area of 25 ha. A full description of the model is given in Wang and Grimm (2007) using the standard format for describing individual-based models, ODD (Overview, Design concepts, Details; Grimm et al. 2006, 2010). The model allows the fate of each individual and its territory to be followed, day by day, in heterogeneous landscapes consisting of different habitat types (Fig. 1).

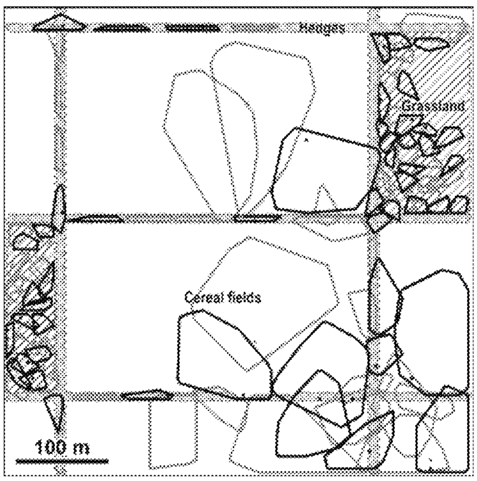

Figure 1. Output of an individual-based model of the common shrew (Wang and Grimm 2007) on a certain day of the simulation. Black lines delineate home ranges of males, gray lines of females. Home ranges in cereal fields need to be larger than in grassland or hedges because of lower resource levels. Home ranges are drawn as minimum convex polygons by connecting the outmost cells occupied by their owners (from Wang and Grimm 2007).

Parameters affecting home range sizes were calibrated to match observations of a certain field study. Likewise, daily mortality was calibrated to obtain populations able to persist in good habitats. All other model parameters were taken from field studies. To make sure that the model captures important features of the internal organization of real populations of the common shrew, it was compared to multiple patterns observed in reality („pattern-oriented modeling"; Grimm et al. 2005, Grimm and Railsback 2005, 2012). Home range size and location varied with season, habitat type, and shrew density qualitatively similar to what is known from the field. Further patterns successfully tested were: proportion of pregnant and lactating females and the age distribution of juveniles and subadults. Thus, although the model certainly is not realistic in the sense that it takes into account all aspects of real populations, it is realistic enough to qualitatively predict the response of populations to additional mortality.

Accordingly, Wang and Grimm (2010) explore various hypothetical scenarios by applying pesticide-induced mortality on either April 1 or July 15: on that day, all individuals had an additional probability of 10 or 20% of dying. They contrasted orchards with and without 10 or 20% hedges, and compared different endpoints such as population size, daily population growth rate, recovery time, and extinction risk. They found that population size is more sensitive for detecting short-term effects than population growth rates and that landscape structure and timing of application had strong impacts on population recovery. For example, with 20% additional mortality on April 1, the population stabilized in orchards including 20% hedges, but continually declined in landscapes without hedges (Fig. 2).

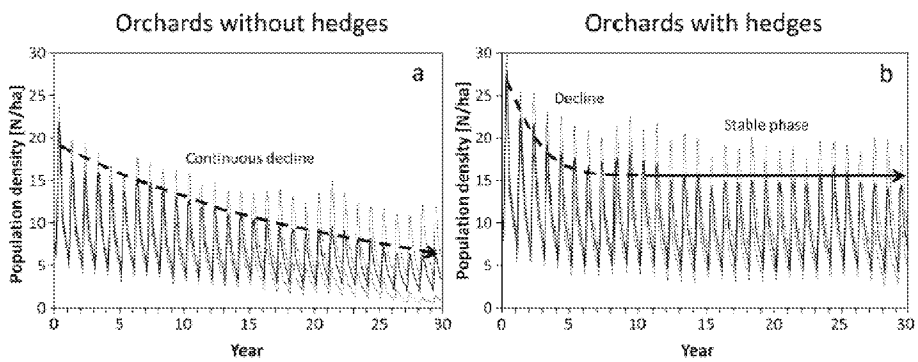

Figure 2. Population dynamics in orchards with and without 20% hedges with a yearly application of 20% additional mortality on April 1 (from Wang and Grimm 2010).

The model of Wang and Grimm (2007, 2010) can in principle be used for regulatory higher tier risk assessments of small mammals. Its main limitation is that only few

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empirical studies exist that can be used for parameterizing, testing, and validating the model. But it clearly demonstrates the potential of well-tested ecological models for risk assessment of pesticides. A further exemplary demonstration of this potential can be found in Topping et al. (2009), who analyze, using much more detailed models, scenarios including skylarks, beetles, spiders, and field voles. Galic et al. (2010) give an overview of the types of insights for ecological risk assessment that can be gained from population models, which are all based on population models' ability to assess population status after integrating lethal and sublethal effects including behavioral changes, at the individual level.

Rationale and Approaches of Mechanistic Effect Modeling

Ecological models have to be based on conceptual models which reflect our current understanding of the system represented in the model. Conceptual models are usually formulated verbally or graphically, which by itself provides no means for testing whether they are consistent and complete. Modelers therefore use formal notations, based on mathematics and computer logics, to translate conceptual models into a framework that allow rigorous calculation of their consequences. Ecological models are thus, broadly speaking, tools for studying if-then scenarios: *if* we agree on a certain set of simplifying assumptions, *then* we have to accept the consequences predicted by the model.
At the beginning of modeling projects, we are usually unhappy with their consequences because they do not match observations, so we revise our assumptions. Model development is therefore an iterative process (Fig. X).

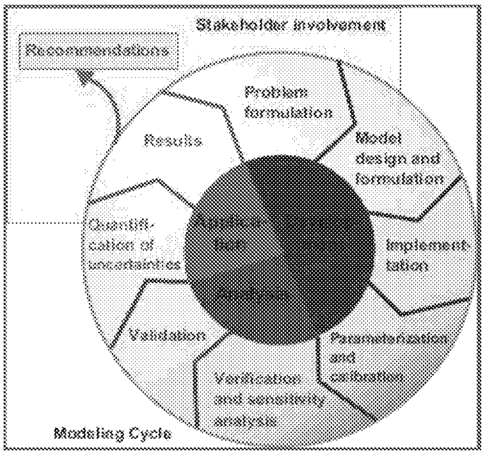

Figure X. Tasks of the „Modeling Cycle", i.e. of the iterative process of formulating, implementing, testing, and analyzing ecological models (after Schmolke et al. 2010b). Full cycles usually include a large number of subcycles, for example verification leading to further effort for parameterization or reformulation of the model. The elements of this cycle are used to structure a new standard format for documenting model development, testing, analysis, and application for environmental decision making, TRACE (Schmolke et al. 2010b).

The Modeling Cycle" depicted in Fig. 3 is relevant for any type of model, but many different types of model design and formulation exist (Schmolke et al. 2010a). Simple models, which are formulated via one or a few coupled differential equations, keep track of the processes causing changes in population size, such as mortality, reproduction, disturbances, etc. They are easy to communicate and understand but usually too poor in structure and mechanisms to be predictive and testable. Matrix models go beyond population size and consider the age, size, or stage structure of populations. They are frequently used to predict population growth rate and the sensitivity of growth rate to changes in mortality or reproduction of certain classes of individuals. Again, matrix models are easy to communicate but once they are designed to include stochasticity, spatial effects, or density dependence, they have to be run on computers and are therefore no longer very different from individual-based models (IBMs). Simple matrix models have a standard format and are relatively easy to parameterize and analyze. They project current average conditions into the future and can therefore be used for initial screening, corresponding to lower tier tests in risk assessment, with small or negative population growth rate indicating risk.

IBMs are computer simulation models in which each individual and its life cycle is represented explicitly (see the common shrew model presented above). Population dynamics and growth rates emerge from what individuals do and how they interact with each other and their environment. IBMs are harder to communicate, parameterize, test and understand than simpler mathematical models, but nevertheless used when one or more of the following factors are assumed to be essential for explaining population dynamics: local interactions, differences among individuals, and adaptive behavior (Grimm and Railsback 2005). IBMs are no longer new but routinely used not only in ecology but also in many other disciplines ranging from behavioral ecology to social sciences, where they are usually referred to as "agent-based" models (Railsback and Grimm 2012). Strategies exist to optimize model complexity (Grimm et al. 2005) and to formulate and communicate IBMs according to a standard format, the ODD ("Overview, Design concepts, Details") protocol (Grimm et al. 2006, 2010).

To use models for pesticide risk assessment, two conflicting criteria for assessing the suitability of models are critical: on the one hand, models need to be complex enough to deliver testable predictions which enable decisions about whether or not the model is a sufficiently good representation of its real counterpart. On the other hand, models need to be simple enough to be thoroughly analyzed and fully understood. Modeling thus requires finding the optimal level of model complexity (Grimm et al. 2005, Grimm and Railsback 2012).

Understanding the main process within a model is decisive, otherwise we would be asking for blind faith in output from the equivalent of a black box. For some questions, simpler models can be sufficient, correctly predicting trends and general mechanisms without making quantitative predictions. For other questions, more accurate predictions are required, which is possible if the models are driven by first principles, such as physiology, stoichiometry, or adaptive behavior, and if enough data are available to directly or indirectly estimate model parameters with sufficient certainty. Highly ecological predictive models have been developed (e.g., Railsback and Harvey 2002, Stillman and Goss-Custard 2010, Topping et al. 2009), but all required more than five person years before first versions could be used to support decision making. However, once a predictive model exists, it pays off extremely well because it can then be used as a virtual laboratory to answer a wide range of questions regarding population dynamics under different and possibly new environmental conditions.

Modeling Practice for Risk Assessment

Claims about the high potential of ecological modeling for pesticide risk assessment are not new and have been made for at least 20 years (Barnthouse 1992). In fact, approximately one hundred academic publications exist that use population or other ecological models to explore the effects of pesticides at the population level (Schmolke et al. 2010a). Galic et al. (2010) summarize the scientific insights of these studies, which are certainly important and contribute to our understanding of the significance of individual-level effects at the population level. Nevertheless, in Europe so far population models have not been used, with very few recent exceptions, for regulatory risk assessment and this seems to be similar in North America. Why is this so? Schmolke et al. (2010a) found that most models in this field are not fit for being used for pesticide registrations. The main reason is that criteria for being accepted as a scientific publication, such as novelty, focusing on one main aspect, simplicity, or generality, are less relevant for making a model suitable for basing environmental decisions on their output. In most cases, choice of model structure and complexity was not justified, endpoints directly relevant for regulatory risk assessments were not considered, sources of parameter values were unclear, uncertainty of model output was not communicated, and – most importantly – little effort was made to prove that the model was a sufficiently good representation of the real population such that insights gained in the model world could be transferred to the real world with sufficient confidence.

This situation is, however, changing in Europe. Two main challenges to make models fit to be used for regulatory risk assessment are (1) the establishment of Good Modeling Practice (GMoP), so that both industry and regulators have clear criteria for how to create and assess models, and (2) the lack of researchers who are well-trained both in ecological modeling and risk assessment (Thorbek et al. 2010). Therefore, CREAM, a large research and training network funded by the European Commission, was launched in 2009 (Grimm et al. 2009; http://cream-itn.eu), includes 13 academic institutions and 10 partners from industry, consulting firms, and regulatory authorities, will run until 2013, and will deliver both guidelines for GMoP and more than 20 young researchers trained in modeling and risk assessment. Moreover, models will be developed which, for indicator species and questions, are good demonstrations for how models can be used for regulatory risk assessments.

Elements of GMoP have long been identified but are still widely ignored. The real challenge is to get these elements accepted and used in practice. Schmolke et al. (2010b) found that for this, regulators or, more generally, decision makers need to be involved, direct benefits for modelers who follow GMoP (which usually requires extra effort) need to identified, and a consistent terminology needs to be established. Therefore, the basic approach of CREAM to establish GMoP is to define and use a standardized documentation framework, TRACE (TRAnspararent and Comprehensives Ecological Modeling). Schmolke et al. (2010b) suggest the use of the structure of the iterative modeling cycle (Fig. 3) as the basis for a general and standardized document structure. As a result, all models that are to be used to support pesticide registration and come with a TRACE documentation as a supplementary document, can be assessed in exactly the same way. Regulators will know that, for example, sensitivity analysis will be described in Section 2.2, the conceptual model underlying the model's design can be found in Section 1.2, etc. Modelers, on the other hand, will know that regulators will expect to see, for example, a documentation of sensitivity analysis, at some point, so they can use the TRACE format as a checklist. The direct benefit for the modeler is that the TRACE format helps keeping notes in the "modeling notebook", which corresponds to "lab journals" in laboratories, in a format that later can directly be transferred to TRACE documents.

Once, by the end of the CREAM project, a critical number of example TRACE documents exist, more specific assessment guidelines can be developed that help standardize the use of ecological models for regulatory risk assessment. This includes the agreement on standard scenarios, species, landscapes, ecoregions, and population-level endpoints. Honeybees and pollinators will play an important role in this context, due to their unique significance for biodiversity and ecosystem services.

Existing Models of Pollinators

Quite a few models exist that address various aspects of honeybee behavior and ecology (for an overview, see section 5.4. in Schmickl and Crailsheim 2007). However, there are surprisingly few sufficiently described models addressing dynamics of non-swarming, managed colonies which include the full life cycle of worker bees from a single hive over several years such that colony-level effects can be assessed (Table X).

Table 1. Colony models that include the full life cycle of worker bees and run long enough, i.e. two or more years, to assess status and survival of a model colony. The third column lists additional factors included in the model that can affect colony status and survival.

Table X

Reference	Purpose of model/Question addressed	Additional factors
Omholt (1986)	Explain brood-rearing peaks in non-swarming colonies	
DeGrandi-Hoffman et al. (1989)	Simulate honeybee population dynamics to support management	
Martin (2001)	Explain the link between varroa mite infestation and honeybee colony collapse, including the effects of virus diseases	Varroa and virus infections
Al Ghamdi and Hoopingarner (2004)	Develop a tool for research and management; interaction between varroa and honeybees	Varroa
Thompson et al. (2005), (2007)	Explore effect of an insecticide on colony dynamics	Pesticides
Schmickl and Crailsheim (2007)	Explore significance of important feedback loops, pollen supply, and brood cannibalism	Swarming
Becher et al. (2010)	Does temperature during development affect colony survival?	

Khoury et al. (2011)	Impact of increased forager mortality on colony growth and development	

Two of these models are interesting from an academic point of view, but too simple to be tested against observed data (Omholt 1986, Khoury et al. 2011). Nevertheless, theoretical insights can guide the design and analysis of more complex models. For example, Khoury et al. (2011) implement two feedback mechanisms: between colony size and brood production and between the number of foragers and recruitment to foraging, which have been referred to as "social inhibition" (Leoncini et al. 2004). They found that if forager mortality exceeds a certain threshold, the colony can no longer maintain itself and will decline to extinction. These feedback mechanisms have been observed empirically and the results of Khoury at al. (2011) suggest that their significance should be further tested in more detailed models, containing a colony's age structure, further feedback mechanisms, and variable environmental drivers.

The model by Thompson et al. (2005, 2007) is also simple and considers the abundance of brood, in-hive and forager bees. This model was originally used in combination with a more detailed population model of varroa mites (Wilkinson and Smith 2002), but Thompson et al. left out the varroa part and added assumptions about the effects of a certain type of pesticide (insect growth regulators), based on observations from their own experiments. Such re-use of models for new questions can be problematic, since the model's design may not be appropriate for the new questions. In this case, model resolution is likely to be too coarse to make robust predictions, still, the model serves as a demonstration of how, in principle, individual-level effects of pesticides can be included in colony models of honeybees.

The models presented by Martin (2001) and Al Ghamdi and Hoopingarner (2004) are modifications of BEEPOP (DeGrandi-Hoffman et al. 1989), a simulation model proceeding in time steps of one day and representing cohorts (or age classes) of eggs, brood, and adults of both worker bees and drones (Fig. 4). BEEPOP distinguishes between in-hive and foraging bees, whereas the other two models don't. Colony dynamics are driven by the queen's egg-laying rate, which is mainly driven by weather, in particular temperature and photoperiod. Additionally, these models include feedbacks between egg-laying and colony size. Drones are mainly included because mites are more attracted by drone cells and mite reproduction is higher in drone cells, so that the proportion of drone cells has an important impact on the dynamics and effects of varroa infestation.

BEEPOP has been augmented by detailed modules for including effects of pesticides (Bromenshenk et al. 1991). The module BEETOX included a toxicity database for more than 400 chemicals and calculated lethal and sub-lethal effects for specific exposures; the module BEEKILL allowed to link these effects to exposure scenarios and feed the resulting changes in mortality, development and longevity into the colony model. Unfortunately, details of these modules were not published and the software implementing them, PC BEEPOP, is unlikely to run on modern computers. It also seems that it has never been used for regulatory risk assessment of pesticides, probably because it was very much ahead of its time. Nevertheless, the design of PC BEEPOP is interesting since it allows to test effects of pesticides on honeybee colonies in a standardized way. Becher et al. (2010) include the effect of colony size and structure on heating and the resulting temperature in brood chamber. It had been observed that brood developed under higher temperatures proceeds faster from in-hive tasks to foraging. It turned out, however, that this has little effect on the dynamics and status of the colony. This is a good example of the role of models for relating individual-level effects to colony-level phenomena. Without the model, it would have been impossible to predict this relationship for the temperature effect, simply because colony structure, environmental drivers, and feedback mechanisms are too complex to be even qualitatively assessed just by reasoning. Negative results, as in this case, i.e. the working hypothesis is shown to be false, are no less important than positive results.

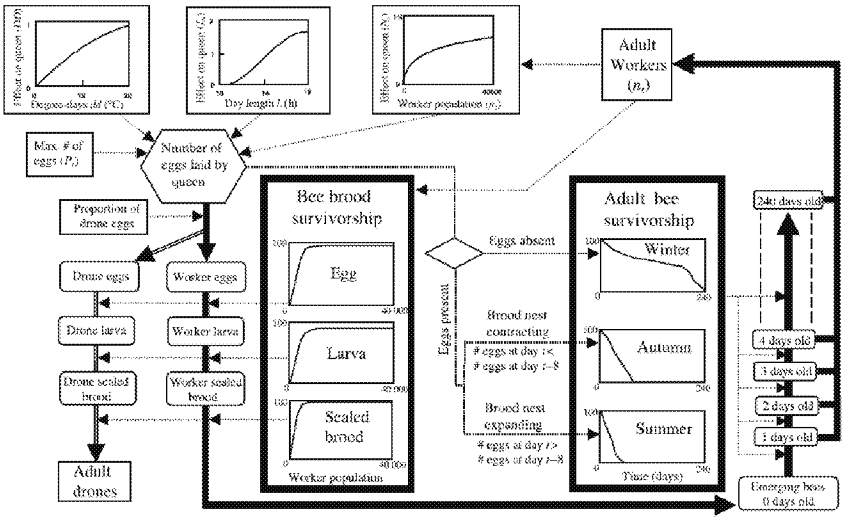

Figure X. Conceptual diagram of the colony model of Martin (2001). Solid lines represent the flow of individuals between developmental stages and dotted lines represent influences (from Martin 2001).

The most complex colony model is HoPoMo (Schmickl and Crailsheim 2007). In contrast to all other colony models, HoPoMo is not entirely driven by demographic rates, such as egg-laying rate of the queen and age- and task-dependent mortalities. Rather, the current number, stage, age, and task of bees are used to calculate the estimated requirements of the colony for nectar and pollen. Depending on current stocks of these two resources, the proportion of worker bees devoted to different tasks is dynamically reallocated every day. The three different tasks distinguished are nursing, food processing, and foraging. HoPoMo includes a large number of further feedbacks between the current state of the colony, or parts of it, and process rates.

HoPoMo consists of 60 difference equations, which are all well documented and biologically justified. The model has been thoroughly tested, including sensitivity analyses and exploration of certain mechanisms. It reproduces several empirical patterns and correctly predicts at least one feature of real colonies that was not used to calibrate or design the model, but emerged during analysis of the full model: in smaller model colonies, with no more than 20,000 brood cells, the number of unsealed brood cells shows oscillations similar to what has been observed in real experimental hives. The model has, however, not yet been used to answer any specific question about how colonies respond to environmental stress.

Two of the colony models in Table 1 also consider infestation with varroa mites. Phoretic mites, i.e. mites attached to worker bees, enter brood cells about one day before they are sealed, and reproduce within these brood cells. Emerging mites try to infest another brood cell or become phoretic, and thereby spread varroa infestation. During the interaction with brood and worker bees, mites transfer viruses, for example Deformed Wing Virus (DWV), or Acute Paralysis Virus (APV). The model of Martin (2001) integrates honeybee and mite population dynamics and the effects of viruses. It shows, for example, that the less virulent DWV will become more widely spread than APV, and that mite control measures need to be taken before the longer-lived overwintering bees emerge. Further varroa models, which focus on various aspects of varroa population dynamics, but are coupled to much simpler colony models than BEEPOP, include Omholt and Crailsheim (1991), Fries et al. (1994), Martin (1998), Calis et al. (1999), Wilkinson and Smith (2002), and DeGrandi-Hoffman and Curry (2005).
For the purpose of pesticide registration, it seems necessary to use models that allow inclusion of varroa infestation because, at least in Europe and North America, varroa is an ubiquitous stressor. It remains an open question, though, in what way varroa infestation could or should be taken into account for pesticide registration. Should decisions be made to ensure safety under a worst-case assumption of high infestation where colonies have high risk of collapsing even without exposure, under an assumption of effective varroa management by beekeepers, or should average infestation levels based on national or international surveys be used? These questions cannot be answered scientifically, but robust, well-tested, and predictive colony models which allow including varroa and possibly other stressors would support decisions by quantitative arguments. Currently, only the model by Martin (2001) is suitable for this purpose. On the other hand, HoPoMo is a more realistic model and includes feedback mechanisms which seem to be important for the functioning of a colony; in particular, HoPoMo is driven by pollen and nectar stores, demand, and availability in the landscape. If HoPoMo

would include a module representing varroa infestation and virus effects, it would currently be the most suitable model for pesticide risk assessment. However, changes in landscape structure, crop plants and their rotation, and agricultural practice also affect honeybee colony performance so that, for registration purposes, a model should also allow such factors to be represented with sufficient detail regarding spatial structure, crop dynamics and rotation, and foraging behavior. Adding such a module to HoPoMo would make an already very complex model even more complex and therefore harder to test and understand. Therefore, a colony model that includes varroa, viruses, and foraging in heterogeneous landscapes should preferably be similar in design to the model of Martin (2001) but include the most important feedbacks included in HoPoMo.

A well-tested prototype of such a model, dubbed "BEEHAVE", was developed by M. Becher and co-workers at Rothamsted Research, UK, in 2011. Its purpose is not pesticide registration per se, but to explore the possible reasons for honeybee decline and collapse. For this purpose, the model includes varroa, viruses, and explicit foraging in heterogeneous landscapes. The option to include pesticide effects, or other additional stressors subsequently shown to be important, was considered from the beginning of this modeling project and a design developed to enable this to be relatively straightforward. The model and its computer code will be made available in 2012, so that other researchers can test the model independently and use or the model for various purposes.

As for non-*Apis* pollinators, fewer models exist than for honeybees. The population model of the solitary red mason bee, *Osmia rufa* (L.) (Ulbrich and Seidelmann 2000) shows, however, that if sufficient empirical knowledge of a species' ecology and behavior exists, developing a population model is straightforward and can lead to important insights. The purpose of the *Osmia* model was predicting the risk of extinction of this solitary species in different types of habitat, which are characterized by the amount and quality of food they provide. The model is individual-based and focuses on cell construction and maternal investment in brood cells. The life stages distinguished are eggs, larvae, imagines in cocoons, males, pre-nesting females, and nesting females. A key decision of nesting females is the sex determination of their brood. The first brood cells are always daughter cells but at some point the mother bee switches to construction of son cells. In the model it is assumed that this switching depends on the mother's weight,

i.e. heavier bees produce more daughter cells. Likewise, size of progeny is related to their mother's weight. As a measure of habitat quality, time for cell construction was used as a proxy (Gathman 1998). In this way the model can be linked to habitat quality without explicitly representing habitat and foraging. As stressor, parasites were taken into account, with parasitism rates being higher for longer cell construction times. Mean population size and extinction risk were taken as population-level endpoints.
Mitesser et al. (2006) developed a colony model for the halictid bee *Lasioglossum malachurum* to explore the emergence of activity cycles, which are typical for some annual eusocial "sweat bees" (Halictidae). The model is very simple and includes only two state variables, the numbers of workers and of sexuals; the time horizon considered is so short that mortality of sexuals could be ignored. Still, there is no principle reason why it should not be possible to develop an age-structured model, similar to BEEPOP or BEEHAVE that includes the full life cycle.

A very interesting individual-based model of bumblebees was developed by Hogeweg and Hesper (1983). It includes the full life cycle of individuals, different types of behaviors, and is, like HoPoMo, to a large degree driven by food collection and consumption and time budgets for certain activities. Focus, though, is less on colony dynamics per se but on explaining division of labor within the colony and so-called "dominance interactions", by which this division emerges. This model was about 20 years ahead of its time as individual-based models, which go beyond demographic rates and include behavior, have only become more widely used within the last 10 years. It would certainly be worthwhile to re-implement this model and try to adapt it to new questions. Whether or not it would be sufficient to just assume division of labor, or have mechanisms included that allow this division to emerge, remains an open question.
In general, eusocial non-*Apis* pollinators have simpler and smaller colonies. This implies that, although they benefit from cooperative activities, they do not maintain buffer mechanisms and reserves which would be as powerful as in honeybee colonies. They also show greater foraging activity, to compensate for the lack of maintained reserves, potentially increasing risk of pesticide exposure.

A bottleneck for developing models for non-*Apis* pollinators might be the lack of data about their foraging behavior in real landscapes since exposure to pesticides to a large extent depends on foraging. Detailed foraging models need to be developed and parameterized and tested using corresponding field studies and experiments (J. Everaars, *unpubl. manuscript*).

Discussion

Sophisticated tests and schemes exist to assess the risk that pesticides impose to honeybees. Current regulations and thresholds seem to be conservative but still leave many questions open. The problem is that without performing controlled, long-term experiments with colonies in real landscapes, exposed not only to pesticides but also other stressors (including beekeeping practices), we cannot be certain whether or not a sublethal or lethal effect of pesticides observed in laboratories or field experiments implies an unacceptable risk to the functioning and survival of a colony. For example, if on a normal day an average of 100 dead bees is found around the hive, and during acute pesticide exposure 300 dead bees are found, is this of any significance to the colony? Likewise, if larvae develop more slowly, or worker bees have a shortened lifespan due to pesticides, how does this affect colony functioning in terms of honey production and pollination? Answering such questions with real experiments might be possible to some degree, but would require enormous resources.

Ecological models could, in principle, compensate for this limitation of empirical approaches. And there are, indeed, fields where models are used to support environmental decision making. For example, recent regulations of wildlife diseases, such as rabies or classical swine fever, are based on predictions of models which are quite similar to the common shrew model presented above (Thulke and Grimm 2010). In some federal states of Germany, forest management plans on the time scale of 10 – 20 years are based on predictions of the individual-based forest model SILVA (Pretzsch et al. 2002). Common features of these and other ecological models used for decision making is that their development took at least five years, and their acceptance by the responsible decision makers about 10 years.

Establishing the use of ecological models to assess risk of pollinators, in particular honeybees, can nevertheless be achieved faster. Well-tested and documented models already exist, which can at least be used, preferably in joint workshops, to discuss and learn the use of such models for higher-tier risk assessments. BEEHAVE, the model currently developed in the UK, holds further promise, in particular because it includes the main potential stressors of colonies and foraging in heterogeneous landscapes. Ideally, to make BEEHAVE fit for use with pesticide registrations, it would need to be used in one or more workshops where researchers from all three sectors involved in pesticide risk assessment, industry, regulators, and academia, agree on standard model scenarios, endpoints, and risk assessment schemes. BEEHAVE is described in a standard format (ODD, Grimm et al. 2006, 2010), its development and analysis will be available as a TRACE document, and it is implemented in a software platform, NetLogo (Wilensky 1999), that is freely available and easy to learn. BEEHAVE is thus designed to be tested, used, and developed not only by its developers but by the scientific and user community involved in honeybee research and management.

The good news is that honeybee models are less limited by data for parameterization than models of most other species. Experimental managed colonies are relatively easy to observe in the laboratory and field, bee behavior has been investigated a lot, and beekeepers accumulated sound empirical knowledge on how colonies respond to environmental events and beekeeping practices. Foraging still is a bottleneck in empirical knowledge, but remote sensing techniques can be used now to follow the flight path of individual foragers (Riley et al. 1996, Osborne et al. 1999). Moreover, in response to the decline or collapse of honeybees in Europe and North America, large international networks like COLOSS (Neumann and Carreck 2010) compile and analyze huge amounts of data, which can be used to test model predictions.

Ecological models are no silver bullet to solve all problems of pollinator risk assessment, but they are a valuable and needed tool for extrapolating individual-level effects to the colony-level, to overcome important limitations of field studies, and to explore endpoints that quantify adverse effects not only on pollinators per se but also on biodiversity and ecosystem services.

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CHAPTER 12 RISK MITIGATION AND PERFORMANCE CRITERIA FOR RISK MANAGEMENT

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The Role of Risk Management in Pollinator Protection

The risk assessment paradigm discussed at the SETAC Pellston Workshop articulates a process to measure the effects of a compound against the protection goals of a regulatory authority. When sufficient data are available to reasonably predict that the intended use of a plant protection product is inconsistent with protection goals of a regulatory authority, and the use of that product remains beneficial and desirable to stakeholders, then risk managers may seek to either continue to refine the estimate of risk, through higher tier testing/analyses (if this remains an option), or to bring the estimated risks into line with the protection goals through specific mitigation measures affecting the proposed use of that compound. Regulatory agencies rely upon management techniques to balance environmental protection goals with other (stakeholder) demands. Consequently, the role of mitigation is central to the process for pesticide regulation. With the exception of few scenarios[82], most mitigation includes reducing potential exposure. The regulatory agency may mitigate the potential risk by denying use on a particular crop or use site. However, in most cases, mitigation actions are those which modify the manner in which a product is used.

Stakeholders in the process of risk management include regulatory agencies (national and local), chemical producers, distributors, field advisors, and practitioners (including growers and applicators). At the national level, regulatory authorities are charged with registering pesticide products in a manner consistent with their statutory responsibilities. At the local level, *e.g.*, state governments in the US, have their own pesticide registration process, which is equally or more protective than the national level. In other scenarios, in

[82] Certain inert ingredients have been shown to [indirectly] increase the potency of a compound; in addition, specific environmental conditions may also modify the behavior, and therefore the potency of a compound.

France for example, specific restrictions can be implemented based on specific cropping or pedo-climatic conditions that may be associated with increased potential res,. At the field level, (additional) mitigation actions can be developed, promoted and implemented by industry experts, crop specialists, beekeepers, growers and/or pesticide applicators that extend beyond what is legally required by the regulatory authorities.

Mitigation language should be specified in a way that allows for consistent (spatial and temporal) implementation. If mitigation language fails to be clear enough for proper, consistent implementation, then inconsistent protection scenarios may result, and the relationship between the regulatory decision and the protection goals may be lost. Clarity and consistent interpretation is also important because the use of a pesticide product inconsistent with the label directions is in many countries considered a violation of the law that may carry with it prosecutorial action. Insofar that the adjudication of the label violation involves investigation by a third party (usually a local regulatory authority such as in the US) and arbitration by a civil official, the clarity of the intended use and restrictions associated with a product label is necessary in order to establish misuse. Misuse of a pesticide can also result in severe adverse effects on either human health or the environment.

Regulatory authorities directly or indirectly rely upon feedback information to understand whether assessments and decisions actually support stated protection goals. Feedback information may come in different forms, such as research studies, reports of bee poisoning incidents, or targeted monitoring programs. Feedback information can provide insight into how a product is actually used, unforeseen variables that affect the use of a compound, unforeseen effects of a mitigation action, and/or simply whether the mitigation measures are sufficient to ensure the protection goal(s). Targeted programs (*i.e.*, investigation designs that time information collection with the actual use of the products), can be expensive but provide high quality data. Investigations, such as eco-epidemiological analyses[83] may not be as valuable as targeted monitoring programs, but can provide information on one or several co-variables. Information gained through bee

[83] Eco-epidemiological analyses are….

poisoning incident reports may lack some information (such as timing of application, application rate, or analytical analysis) that may be useful in establishing that a particular chemical use resulted in an incident, but may provide information on a specific type of product or use scenario that may be anecdotally linked to an incident. In addition, because incident reports frequently rely upon volunteer reporting, it is difficult to know the degree to which incident reports reflect real world conditions. Therefore, a lack of incident reports may or may not be indicative of safe, intended use of products, or conversely may not represent the extent of events related to a product, *i.e.*, the absence of incident reports cannot be reasonable construed as the absence of incidents. Conversly, the presence of isolated incidents may not necessarilly indicate a potential risk issue with a product. However, a pattern of incidents with relation to a specific compound, application method, crop, etc, a potential risk may be a clear indication of a risk issue. Nonetheless, information from these feedback sources provides multiple lines of evidence which can be used to inform and modify existing or future assessment or management decisions. (Additional discussion may be found in a recent European (OPERA) review (Alix et. al., 2011)

Below is a brief discussion of considerations with respect to risk management for *Apis* and non-*Apis* bees.

Regulatory Risk Mitigation Methods

The risk assessment should provide a clear description of the risk (*i.e.*, the likelihood and magnitude of an adverse effect) that needs to be mitigated; this is the first step to developing the appropriate management actions. Knowledge of the chemical physical properties, environmental fate and ecological effects of a compound are integrated with the use, of a compound to provide the information necessary to develop potential management options. Specific characteristics of the risk(s) to be mitigated may include the following.

- Whether the risk is related to acute effects on adult bees, chronic effects on adult bees, adverse effects on larval development, or other effects (such as interactive effects of tank-mixes containing insecticides and fungicides).
- Whether the risk is related to honey bees, other species of bees, or both.
- Whether the risk is related to a particular crop or site being treated, to off-target movement of the pesticide to adjacent crops or blooming weeds where bees may be foraging on nectar and/or pollen, or to other concerns (such as contamination of nesting materials used by non-*Apis* bees).
- Whether the risk is related to a particular application mode (systemic or topical) or method (such as spray, or irrigation)
- Whether, (such as in the US), the pesticide has an extended residual hazard to bees (lethal to 25% of exposed bees for more than 8 hours).

a) <u>Crops Requiring Pollination by Bees:</u> Central to managing risk of pesticides to bees is controlling potential exposure at the time, or under conditions when bees are [likely to be] present in an agricultural setting. One of the most critical issues for risk management is when bees are present at a site for pollination of the crop (Riedl *et al*., 2006), which may also include bees foraging on understory bloom or on an adjacent or border area. For crops that require pollination by bees, the primary consideration should be to protect bees from pesticide residues that represent a hazard potential. While every attempt should be made to avoid applications of insecticides and fungicides during the pollination period, use of a plant protection product may be needed (or designed for use) when the crop may be most attractive to bees. When developing risk mitigation statements, there are several management options that could be considered:

- <u>Product Formulation:</u> Typically there may be several formulations that could be used to treat a crop/pest combination. To the extent possible, formulations should be those that pose the least threat to bees. Formulations that approximate pollen grains (*e.g.*, some microencapsulated products) in terms of particle size can lead to greater exposure as bees may accumulate the product through their normal foraging activity; however, addition of a sticking agent to a foliar application can potentially reduce transfer from the plant to the bee. Granular formulations are typically considered the least hazardous to bees. Seed treatments also provide limited exposure (similar to granular formulations) provided that dust (from abrasion during planting) emission is properly managed. However, dust particles from seed treatments were responsible for a large number of bee poisoning incidents in Germany during 2008 (Pistorius *et al.* 2009).) Soluble and emulsifiable (liquid) formulations are usually safer to bees than wettable powders. Dust and micro-encapsulated formulations may be more hazardous to bees than other formulations, (or routes). For more information on the relative hazard of different formulations, see Johansen and Mayer 1990.

- Method of Application: The application method may also be examined to reduce potential environmental exposure. Generally, ground applications result in less off-target drift to both adjacent areas and the understory than aerial applications. Soil incorporated application methods provide limited environmental exposure (via drift); however since the compound is available to all the growth material, this method may lead to pesticide residues to be expressed in understory bloom. With respect to aerial application, droplet size can have a marked effect on the extent of drift; in general, larger droplet size is less likely to drift compared to finer droplets.

- Application Parameters: Limiting the use rate and frequency of application to the minimum required to effectively control the pest or disease organism.

- Understory and Adjacent Areas: Understory can be a source of either foliar (*e.g.*, from aerial drift) or systemic (when pesticide residues in the soil are taken up by understory flora) exposure to pesticides applied on field. Note that the understory may represent an attractive source of nutrition for the bees separate from, or in addition to, the cultivated crop. Potential methods of controlling weed bloom include mowing, disking, flailing, or through use of an herbicide. However, it should be noted that measures to eliminate understory in doing so, it is important to note that alternative forage and habitate areas (which may provide nutritional diversity) for both pollinators and arthropod fauna are then forfeited. (Not considered a sustainable mitigation measure in some European countries.)

- Application Timing and Environmental Conditions: Applications may be restricted to times when bee activity is expected to be at a minimum. Honey bees don't forage at night (in temperate regions), and don't begin actively foraging until the temperature reaches at least 55°F (12.8°C). This risk mitigation technique is only effective if the pesticide has an intermediate residual hazard to bees of 8 hours or less (evening applications only), has a short residual hazard of less than 4 hours (evening or morning applications), or if flowers are closed during applications. It should be noted though, that different species have slightly different activity times; and, high temperatures encourage bees to forage earlier in the day or continue to forage later into the evening than usual. Late evening applications are generally less hazardous to bees than early morning applications; environmental conditions such as temperature and dew point may affect the dissipation of a compound, (*e.g.*, slow down), thereby extending a compounds residual toxicity. This management option is likely to be of very limited benefit in tropical regions, since the non-foraging period for honey bees in the tropics is very short when compared with temperate regions. For more information on application timing and environmental conditions, see Johansen and Mayer 1990.

- Tank-Mixes: Tank-mixing may represent an economical option in pest control. However, care should be taken to understand if there are unforeseen effects to non-target organisms from mixing different compounds in a single application. Tank-mixing certain types of compounds may result in interactive effects that can enhance to toxicity of the mixture to bees. (France has recently prohibited tank mixes of triazol fungicides and pyrethroids (JORF, 2010).)

- Notification: Growers may notify beekeepers of anticipated pest control needs. This allows the parties involved to discuss variables and options to reduce potential exposure to bees. While beekeepers may try to protect their stock from an application by covering colonies, doing so for an extended period of time may be damaging to the colonies particularly in warm weather. Further, it may be difficult to move managed bees "on demand" since the configuration of the colonies, number of colonies, and the bee activity level effect how quickly stock can be relocated (or protected). (Also, while moving or protecting may be an option for managed bees, it will not protect non-managed bees.)

b) Crops Not Requiring Pollination by Bees: Pesticide applications to blooming crops, crops with extra-floral nectaries, and pollen shedding crops not requiring pollination that are attractive to bees have also been documented as an important cause of bee poisoning (Riedl *et al.* 2006). The management options listed above should be considered, but the mitigation statements may need to be modified to address the specific circumstances involved with crops that do not require pollination.

Non-Regulatory Risk Mitigation Methods

Where limitations exist with regard to the level of risk management that can be reliably and effectively implemented through a national-scale label (regulatory method), implementation of risk management may be possible at the la*ndscap*e, or field level through best management practices (BMPs) employed by the user (non-regulatory). Alternative or additional methods to mitigate risk to pollinating bees may be used in conjunction with measures identified through the product registration and captured on the product label. Beekeepers, growers, and applicators together with IPM agents, agricultural extension agents, crop advisors and pesticide product representatives can exercise field-level knowledge (*i.e.*, practical experience) to achieve maximum protection for both the grower and the beekeeper. Measures that go beyond the product label reflect local knowledge, and relationships which foster cooperation that are often the most effective way to manage potential risks.

Among regulatory and non-regulatory methods to mitigate potential risks, communication and cooperation between growers, applicators, and beekeepers is perhaps the most important tool to reduce risk, and ensure that the needs of all of the stakeholders are met. Growers and beekeepers engage in reciprocal, mutually beneficial, endeavors; and, it is to the advantage of each to anticipate/respect the concerns/needs of the other. Growers can learn the pollination requirements of the crops they grow, and plan pest control operations with pollination needs in mind. Growers and advisors can proactively manage routine insect pests by developing and monitoring for economic thresholds to initiate appropriate treatment early to reduce pest population and prevent, avoid or lessen loss without having to rely on higher application rates/intervals that may represent a risk to bees. Such a program is often less hazardous to pollinators and other beneficial insects as well. Applicators can use their knowledge of local weather patterns to time applications in a way that responds to pest pressure and accounts for bee activity, and/or chemical physical properties of the pesticide product. Through communication with growers and applicators, beekeepers should be familiar with pest control problems and programs, in order to develop mutually beneficial agreements the better ensure the prudent use of insecticides and fungicides. Beekeepers, growers, crop advisors and applicators should be aware of the toxicity of product(s) being used, and any residual toxicity characteristics. As discussed previously, depending on the size and location of apiaries and weather conditions, some beekeepers can protect honey bee colonies by covering them with wet burlap the night before a crop is treated with an insecticide that has an extended residual hazard. These covers are typically maintained wet and in place for enough time to provide protection from initial hazards. Honey bee colonies should be clearly marked with identification as this facilitates communication.

Apiaries can be situated to isolate them from intensive pesticide application area and to protect them from insecticide and fungicide drift. Establish holding yards for honey bee colonies at least 4 miles from blooming crops being treated with insecticides that are highly toxic to bees.

Ridge tops are preferable to canyon bottoms, as insecticide fines drift down into the canyons and flow with morning wind currents.

Suggested Techniques to Mitigate Risks to Other Species of Bees

Alfalfa leafcutting bees (*Megachile rotundata*) are nearly inactive at 70ºF (21.1ºC) and completely inactive at 60ºF (15.6ºC). Both managed alfalfa leafcutting and bumble bees (*Bombus* spp.) can be safeguarded from potential exposures by removing nests prior to pesticide applications. Shelters for these bees can be built to be covered, closed or removed during insecticide applications to reduce the threat of insecticide drift. Alfalfa leafcutting bees show increased sensitivity to agricultural chemicals after 3 or more weeks in the field; and, should not be replaced into fields until at least one week after treatment with insecticides with an extended residual hazard. Blooms of any type, including weedy species that may be available in adjacent areas on in fence rows, may serve as nesting sites or as a nutritional source for native pollinators (as it is for managed pollinators as well). To the extent that growers can leave such plants undisturbed and manage pesticide drift, they contribute to the conservation of these native pollinators and the diversity of the farm ecosystem. Approximately 70% of native bees are ground nesters, burrowing into areas of well-drained, bare or partially vegetated soil. Growers and beekeepers can provide resources for nesting sites for many native species. More information on improving habitat for native pollinators may be found in Vaughn et al. (2007) and Vaughn and Skinner (2008).

Pesticide Application Technologies to Mitigate Exposure to Bees

Mitigation from exposure to spray applications and drift to off-site areas

For compounds that are acutely toxic to bees by contact exposure and a screening-level risk assessment indicates a potential risk to bees via contact exposure, data from a higher-tier test, such as U.S. EPA's Tier 2 study to evaluate the toxicity of a pesticide on foliage (*e.g.*, alfalfa) should be used to determine when products should not be applied (*e.g.*, Do not apply when bees are actively foraging). To minimize exposure of bees to pesticides, it is important to be aware of weather conditions, particularly wind speed and direction, and avoid applying during those times. Applications at dusk or late evening or early morning prior to dawn when the majority of honey bees are not actively foraging could help minimize contact exposure, depending on the residual time and bioavailability of the pesticide.

Mitigation for exposure to seed treatment dust

In order to minimize the emission of abraded seed treatment dust during sowing, particularly when seeds dressed with insecticides that are toxic to bees, the following parameters are considered to be particularly relevant:

Seed coating quality

Prior to seed treatment, seeds need to be properly cleaned to remove extraneous debris. Thereafter care should be taken to minimize loose dust in the seed bag. The use of optimized seed treatment recipes is a key parameter to guarantee a high abrasion resistance of the treated seed, while for some treated seeds (*e.g.*, corn), the use of appropriate stickers and film-coatings will further enhance the resistance of treated seeds to abrasion.

Seeding technology

When seeds are sown using vacuum pneumatic sowing equipment, the use of deflectors, which direct dust downward into the field being planted, has been demonstrated to reduce off-site dust emission. However, even with deflectors, caution should be taken when using this type of sowing equipment in no-till fields, if blooming weeds are present in the field. In this scenario, dust could be deflected directly onto the flowering weeds. Mechanically operated sowing equipment, as well as those using compressed air, are less prone to emit dust into the environment.

Soil applied uses

Crops that are not in bloom often harbor blooming weeds or have blooming cover crops. These blooming plants may represent a potential source of pesticide exposure for both honey bees and non-*Apis* bees if the plants are exposed to soil-applied systemic pesticides. Chemigation systems should be maintained in proper working order to ensure pesticides will not spray, leak or run-off into areas where potential contamination of blooming plants or water sources for bees could occur. Care should also be taken when making granular applications for the same reasons. These potential routes of exposure are probably best addressed through product stewardship which requires applicator education and post registration monitoring.

IPM / crop rotation

In Europe, seeds coated with systemic pesticide are used in maize (corn) cultivation to protect the plants from soil-dwelling insects. As maize is not a native plant to Europe, attacks from aphids and other sap sucking insects do not represent a significant threat that typically requires pesticide treatment. In addition, IPM techniques can contribute to the natural reduction of soil insects by simply rotating crops. Using these techniques, populations of soil insects can be maintained below detrimental thresholds, thus reducing the need for pesticide treatments, and thus reducing potential exposures to bees.

Landscape management

Preserved habitats, refuges, food resource, *etc*, may reduce the dependence of non-target species on cropped areas. (Vaughn et al., 2007). Variable such as the nature of the refuge, the proportion or density, location and management of such areas contribute to the effectiveness of protected area. Initiatives have been undertaken that illustrate the effect of the implementation of flowering strips on pollinating species (*e.g.*, Operation Pollinator developed by Syngenta, [HYPERLINK "http://www.operationpollinator.com"]) which could provide a useful basis for further recommendations in the future. Further work is needed to actually quantify the benefit in terms of exposure (drift reduction) and impact of the implementation of habitat for native pollinator species. Eventually landscape-level modelling may be used in support of the design of the landscape elements that may be recommended as mitigation measures.

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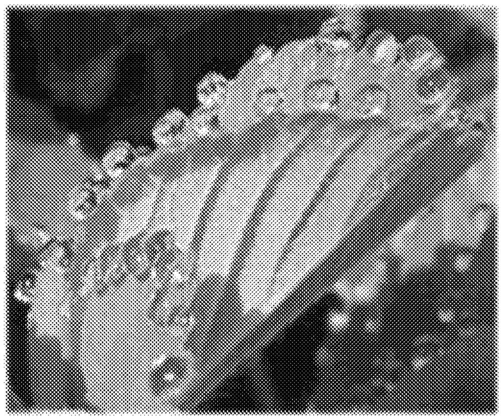
CHAPTER 13 FUTURE RESEARCH NEEDS AND RECOMMENDATIONS

Exposure

Consumption of guttation water as a source of exposure: Various investigations of residues in guttation droplets collected from seed-treated crop plants revealed the potential for high residue levels to be present in guttation droplets (Girolami *et al.*, 2009; Joachimsmeier *et al.*, 2010; Pistorius and Joachimsmeier, 2010; Schenke *et al.*, 2010). Highest residues in guttation water occur immediately after seedling emergence and have been shown to decline with time. Current data suggests that monocotyledons tend to show guttation on a more frequent basis than dicotyledons. Some plants like sugar beets produce practically negligible guttation. If bee hives are located in the immediate proximity to treated crops (field margin), some individual honey bees have been observed collecting guttation droplets. If highly toxic systemic seed treatments or soil applications have been used, some individual forager bees could be potentially exposed lethal levels of residues in guttation water. However, in currently available colony-level studies, neither adverse effects on colonies, nor impact on bee keeping practices have been associated with pesticides in guttation water. Further studies are currently under evaluation, and more research is required to clarify if exposure of systemic pesticides through guttation water needs to be included in the pesticide risk assessment process.



Guttation water on a strawberry leaf. Photograph by Noah Elhardt

Quantify in-hive exposure to larval, queens, and other hive members for use in screening assessments: Data on actual exposure of larvae or other hive members could be established by chemical analysis of larval jelly, royal jelly, and bee bread following a field application (such as in a semi-field or field scenario). Spraying a surrogate crop (e.g., *Phacelia* or buckwheat), enclosed in a tunnel containing a hive with minimal pollen and nectar stores would provide an optimal test system to measure in-hive exposure. Larval jelly and bee bread could be sampled from larval cells and analyzed for the appropriate pesticide residues. Data from a series of such tests that capture a range of mode of actions, application methods etc. could be averaged to provide a generalized value to represent in-hive "pesticide" exposure (*e.g.*, in larval food) for use in screening level analyses. Analysis could include both foliarly applied and systemic compounds. For systemic compounds, representative crops could be selected, and treated using different delivery routes. Residues in leaves, pollen and nectar could be sampled over time, and particularly during bloom to determine uptake and decline rates of the pesticide. This data could help refine the default exposure calculation for systemic compounds. Research such as this would also be helpful in determining the number of samples (*e.g.*, beebread, larval jelly) should be analyzed to obtain a robust and repeatable measurement of residue levels, and would also provide information to compare residue levels in pollen to that in other in-hive products, such as beebread.

Laboratory Studies

Laboratory studies should be conducted that allow direct comparisons between species to different classes of pesticides

Field Studies

Comparisons between Apis and non-Apis species: An obvious knowledge gap identified by the participants of the Workshop is how semi-field and full-field research results on *Apis* translate to non-*Apis* bees. One way to address this uncertainty is to include non-*Apis* bees in semi-field and field studies.

Reliable test for sub-lethal effects: There is a real need for reliable (field-level) tests for sub-lethal effects and a means to translate these effects into meaning measures at the hive level, *i.e.*, to establish quantitative linkages between sub-lethal measurement endpoints on individual bees and more traditional colony-level assessment endpoints. Sub-lethal effects are most often made at the individual level but even when effects are noted it is difficult to extrapolate these effects to the whole colony. Research is needed to develop reliable test measurements to consistently document sub-lethal effects on bee behavior. Equally important, is a means to translate these effects at the individual level to effects at the colony level. Suggestions for sub-lethal tests include: a standard test for foraging disorientation that might include a "time back to the hive" or a maze at the hive entrance.

Determining the degree of adult or brood loss that affects colony productivity and survival: Losses of adult bees in dead bee traps and brood are often noted but the impact of these losses is hard to determine, especially if the losses are transitory. A series of experiments are needed to determine the rate of adult and brood loss necessary to impact colony productivity and pollination and ultimately colony survival. *Apis* colonies have a reserve of worker bees that serve to buffer the effects of temporary losses. However, there remains a fundamental uncertainty regarding the point at which the hives buffer becomes exhausted, and the colony is impaired.

Extrapolating from semi-field or field scale to protection goals: Currently, if any significant effects is observed or measured in a semi-field study, it is predicted that protection goals (as defined above) are unlikely to be met. This is due to inability to confidently extrapolate from effects seen in a semi-field study to what may, or may not occur under field conditions. It would be extremely valuable if research was carried out to link measurement endpoints derived from a semi-field study would result in a protection goal not being met. This may include not only well designed testing, but well designed post monitoring as well.

Recommendations for Future Research in Risk Assessment

From the discussion above, the following immediate recommendations are proposed which aim at further improving the risk assessment scheme that could be developed in these proceedings.

There is a need for cost-effective reporting schemes that provide incentives to all parties involved, *e.g.*, beekeepers, applicators, and growers, to help increase accurate representation of use and effects of pesticide use in the field. This information would be an important input to the pesticide regulatory framework (*i.e.*, risk assessment and risk management). Furthermore, a common platform for incident reporting between regulatory authorities would facilitate the sharing of incident data and management strategies.

Modelling has been identified as a promising tool for the purpose of risk assessment and risk management. Further research and work on model development for use in pesticide risk assessment for pollinators would help to document and refine model biological realism, sensitivity, robustness, parameterization and calibration. Models could be used to explore potential linkages between measurement endpoints and assessment endpoints or protection goals. Models could also be used in support of extrapolation in time and space of the outcome of a risk assessment based on laboratory studies. Models could also be developed as a support in the design of higher tier studies and landscape management. Collaboration between modellers and others such as regulators, or entomologists would help direct model development and refinement.

The role that landscape management and alternative foraging and habitat resources may play in limiting the impact of pesticides and agronomic practices on pollinators calls for further research in this area. Typically monitoring studies undertaken in agronomic systems proposing diverse options for landscape management would bring this feedback and support appropriate recommendations. Such approaches include population ecology, landscape ecology and exposure characterization. It is noteworthy that the data generated may also feed model development and could thus be generated with the advice of modellers.

Research Areas in Support of Risk Mitigation

Efficacy of risk mitigation techniques in reducing the frequency or severity of bee poisoning incidents. For example, drift reduction technologies and drilling conditions to limit the dust from seed treatment applications, and use of vegetated buffers to mitigate spray drift and provide refuge and habitat for pollinators.

Interactive effects (*e.g.*, synergisism), particularly between insecticides and fungicides. Evidence of interactions have been observed under laboratory conditions, however the relative extend of these interactions in the field remains poorly described. Information on this, including research involving residues occurring in hives is needed to improve our understanding of whether label directions should be revised to restrict or prohibit tank-mixtures of certain pesticides/adjuvants/surfactants that are applied to blooming crops, such as in France for example JORF, 2010.

Interaction between mite control chemicals (acaricides) applied in-hive by beekeepers for control of varroa mites, insecticides and fungicides applied to pollinated crops, and honey bee diseases. Research in this area, in addition to that conducted by the US Department of Agriculture would improve the understanding of whether label use directions for in-hive acaricide applications and/or pesticide applications to blooming crops should be revised.

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